Novel metal chelate and p-ABA affinity membranes for protein isolation

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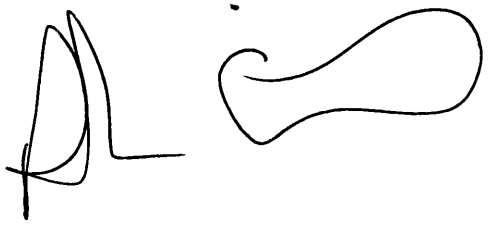
NOVEL METAL CHELATE AND p-ABA AFFINITY MEMBRANES FOR PROTEIN ISOLATION.

submitted by Alan Carter
for the degree of PhD of
the University of Bath 1988



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To my mother and father for the many sacrifices they have made, and to the Jackson and Haberlin families whose friendship and affection I shall always treasure.

(.."I shall forbear to tell you yet precisely
what plans I have for binding you closer
to me in affinity"....)

Tiberius Caesar. AD 27

CONTENTS

Acknowledgment

I would firstly like to express my gratitude to Prof. J.A. Howell for the guidance and encouragement he has given me throughout the course of this project, and also for his warm friendship during my stay at Bath.

I would also like to extend my sincerest best wishes to the postgraduate students, staff and faculty at the University of Bath, and in particular, Dr R Grant, Dr J Hubble and Mr D Powell for their support and technical guidance.

Finally I would like to thank Network for typing this manuscript.

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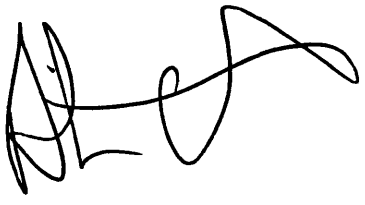
Summary

The development of two types of novel affinity adsorbent constructed from a regenerated cellulose membrane support is described. The first derivative contained p-ABA, a specific serine protease inhibitor. This molecule was covalently coupled to a cellulose membrane, previously treated with CDI, (carbonyldiimidazole) via a hexanoic acid leash. In conjunction with this affinant a metal chelate analogue was manufactured. The metal was introduced onto the surface of the matrix using the chelating reagent IDA (iminodiacetic acid), which was covalently attached to cellulose backbone.

In a series of protein binding studies, a simple model was used to describe the batch adsorption of trypsin onto the p-ABA membranes. The model contained a simple differential rate expression, that approximated the rates of adsorption and desorption of the protein onto the membranes. The values of K_{de} and q_m for the binding reaction were evaluated and then incorporated into an interactive computer simulation programme. This computer package integrated the rate expression, which in turn allowed one to estimate a value for the forward rate constant; k_1. The kinetics of the reaction were modified by changing the initial pH and temperature conditions.

The metal chelate membranes were used to fractionate a range of protein components commonly found in urine and plasma. A tentative identification of the adsorption products from the plasma studies was made using gel electrophoresis. The presence of HSA, transferrin and α_{-1} antitrypsin was indicated by this analysis. In a second investigation the p-ABA membranes were enclosed into a capsule which was then successfully integrated into a hplc unit. This system was used to purify a pretreated commercial sample of urokinase, a protein commonly found in dilute quantities in urine.



Preface

Biotechnology is a highly commercialised industry, which is currently receiving world-wide investment, particularly in the area of downstream processing. Biotechnologists have recognised that the techniques used to extract and purify biological macromolecules, form an inseparable part of the whole biotechnological process. Among the many categories of downstream processing, the conventional filtration techniques such as ion exchange, size exclusion and affinity chromatography are undergoing major reform. This reformation has been prompted by the realisation that the purity of the final product is, to some degree, dictated by the type of isolation method used. In considering the question of purity, affinity chromatography perhaps provides one with the most elegant solution to the problem of purifying individual proteins from complex biological mixtures. The underlying principle of this technique is to covalently bind a suitable affinant to an insoluble support material, that has the ability to selectively and reversibly interact with the component to be purified. Elution can be achieved using any one of a number of procedures which result in the dissociation of the complex. These events will be discussed in greater detail in Chapter I.

A second major category to be included among the diverse repertoire of downstream processes is membrane filtration. This form of separation technique progressed from a phenomena first observed by biologists at the turn of the 18th century, when they recognised that membranes in living organisms had the power to create and maintain concentration differences among substances. A membrane has since been defined as a phase or group of phases that influence the transport of matter between the two uniform phases that it separates. [Synthetic membranes and their applications SERC]. By the nature of most biological processes, the liquid phase is a dilute source of product, which is liable to deterioration if not processed rapidly. The processing speed is subsequently influenced by the form of the separating medium.

Typically, the various types of support available to conventional chromatography tend to be granular or beaded. Unfortunately these materials can suffer from severe chemical and mechanical inadequacies. Soft gels for example, characteristically compress when fast processing conditions are employed. This also induces the formation of channels through the column, allowing the mobile phase to bypass most of the packed bed. Flow rates in these materials are also restricted to a few centimetres/min. A concerted effort is therefore being made to develop novel types of support matrix that have the versitility to withstand the rigorous operational requirements now being imposed upon them.

Although there have been a few advances in the area of matrix development, with the introduction of silicon and polymeric materials [Ohlson 1978, Lowe 1981] the chemical stability and mechanical strength of these derivatives still pose a problem.

It is therefore quite surprising, in light of these difficulties, to find that little attention has been given to the possibility of using a derivatised membrane as an affinity adsorbent. Such a material should be quite capable of processing large volumes of solution with no detrimental effect to the membrane structure and thus effectively overcome the problems encountered with compression and channeling.

The objective of the present study was to develop a suitable affinity matrix constructed out of regenerated cellulose membranes and to investigate their potential as a standard affinity medium.

The introductory chapter of this work highlights the important milestones attained during the respective developments of affinity chromatography and membrane technology throughout this century. This is followed by a description of the techniques and strategies used to prepare two types of protein selective membrane. The theoretical aspects of protein adsorption are reviewed in Chapter III. The theory is illustrated by using a simple model to predict the rate of binding of trypsin onto a highly specific serine protease affinity membrane. The performance of these membranes and suggestions for future work are given in the final chapters.

CHAPTER I : INTRODUCTION TO AFFINITY CHROMATOGRAPHY AND MEMBRANE TECHNOLOGY

AFFINITY CHROMATOGRAPHY

The concept of affinity chromatography is based on the exceptional ability of biologically active substances to bind selectively and reversibly to a complementary bioligand. This phenomena was first documented at the turn of this century when Starkenstein [1910] reported that α-amylase could be selectively adsorbed onto an insoluble starch support. Despite this notable achievement the development of affinity chromatography was substantially retarded, mainly as a result of the complex organic chemistry that was required to synthesise a reliable matrix and the lack of satisfactory methods for immobilising the bioligand. The first method to describe an enzyme purification by means of an insoluble enzyme inhibitor was discussed by Lerman [1953]. It wasn't however until the late 1960's, with the pioneering works of Axen [1967] and Cuatrecasas [1968] in particular, that the full potential of this technique was recognised. Axen reported that biologically active proteins and polypeptides could be coupled to a multitude of polysaccharide materials suitably treated with CNBr. Cuatrecasas demonstrated the selectivity of these adsorbents when he purified α-chymotrypsin and carboxypeptidase using a matrix activated with D-tryptophan methyl ester. It was also in this study that the term affinity chromatography was used for the first time. Although this title is generally used to describe the process of adsorption, the accuracy of this description is questionable. To emphasise the unique biological affinity displayed between the macromolecule and bioligand Porath [1973] introduced the term "Biospecific Adsorption" which was later modified to "Bioselective Adsorption" [Scouten (1974)] which suggested the process was selective rather than specific.

Bioselective adsorption can only be performed successfully when one suitably reproduces, in the conditions chosen for the purification, the interaction between the macromolecule and bioligand in free solution. The investigator must therefore give careful consideration to the choice of bioligand, type of support and method(s) of immobilisation.

THE CHOICE OF BIOLIGAND AND IMMOBILISATION PROCEDURE

The physical and chemical properties of the bioligand will ultimately influence its effectiveness as a biological affinant. Ideally it should form a reversible complex with the component to be isolated, usually having a dissociation constant (K_d) in the range 10^{-4}M - 10^{-8}M. If the dissociation constant is $>$ than 10^{-4} the binding interaction is effectively to weak to achieve a reasonable affinity separation, if on the other hand it is $<$ than 10^{-8} the interaction is so strong that problems occur when one wishes to desorb the protein. It must possess at least one chemically modifiable group that is not involved in the interaction process and it should be soluble in the solvents used to perform the coupling reaction. A selection of complementary ligand systems is given in Table 1.

TABLE 1

LIGAND A	LIGAND B
Antibody	Antigen
Enzymes	Inhibitors
Chelate. Mol.	Metal Ions
Aromatic. Mol.	Dyes
Receptors	Hormones

The choice of immobilisation technique is primarily influenced by the nature of the functional groups on both the bioligand and the matrix. A detailed description of the various methods of immobilisation is beyond the scope of this discussion. In this instance I would like to refer the reader to the reviews by Weetall [1973], Cuatrecasas [1971a], and Dean [1986].

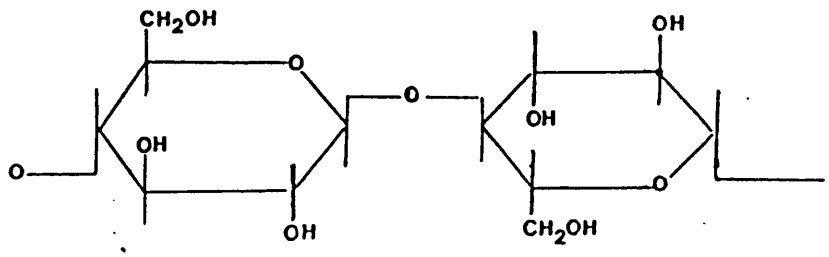
CHOICE OF MATRIX

The problem of selecting a suitable solid support is compounded by the intrinsic characteristics of a gel, which can respond quite unpredictably to the variety of conditions imposed upon it. One of the most important physical features of a matrix is its porosity which effectively influences the flow properties of the medium, [Lowe 1971]. The support must possess an abundant supply of chemically modifiable groups to allow covalent linkage of the bioligand. It must be physically and chemically stable to the conditions chosen for coupling, adsorption and elution, and show resistance to microbial and enzymatic attack. Approximately 90% of all the support materials used in affinity chromatography are derived from a polysaccharide, more commonly, cellulose, agarose or dextran.

CELLULOSE

Cellulose is a natural constituent of plant cell walls. It consists primarily of β-1,4 linked D-glucose units. [Fig 1].

FIGURE 1

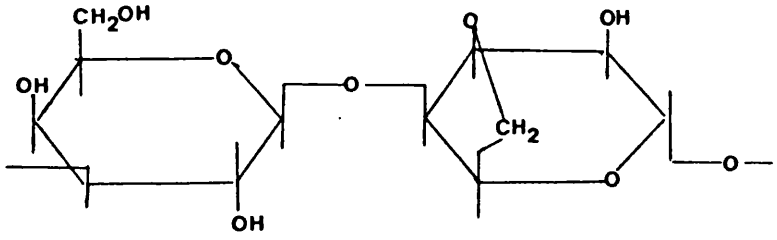


From the pioneering work of Lerman [1953] a plethora of derivatised celluloses have emerged. Those commercially available are generally cross-linked with a bifunctional reagent such as epichlorohydrin. Although in many ways cellulose would appear to have the ideal prerequisites one would expect of a suitable matrix i.e. it has little non-specific adsorption and it is both mechanically and chemically stable, the utility of this material has been limited by the fibrous and non-uniform nature of its structure. This imparts an extremely poor porosity to the medium which prevents the penetration of large macromolecules [Knight 1967].

AGAROSE

By far the most widely employed matrix material is agarose, a linear polysaccharide consisting of alternating residues of D-galactose and 3-anhydrogalactose [Fig 2].

FIGURE 2

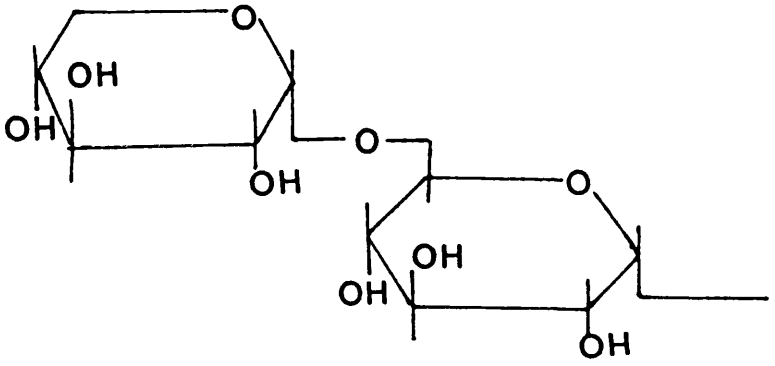


Agarose has an open pore structure and thus exhibits exceptional flow properties [Porath 1976]. Unfortunately in concentrated salt solutions unrefined agarose has a tendency to swell. Many commercial derivatives are cross-linked with epichlorohydrin to enhance their thermal stability. This treatment however negates any advantages originally gained from its open pore distribution.

DEXTRAN

Dextran is a. β-1-6 linked glucose polymer produced as a fermentation product of sucrose [Fig 3].

FIGURE 3



Once again the soluble polymer chains are cross-linked giving a polymer unit that is mechanically quite stable and extremely resistant to chemical attack. Unfortunately the low porosity of these gels has severely limited their use in affinity chromatography.

MEMBRANE TECHNOLOGY

One of the earliest recorded uses of a membrane took place in a monastery in the year 1748 when the friar Abbé Nollet demonstrated the phenomena of osmosis using a semi-permeable barrier constructed out of a pig bladder. Just over one hundred years later Fick received the accolade of manufacturing the first synthetic membrane. Based on the early work of Zsigmonay [1918] Sartorius produced a range of commercial membranes for practical applications in the early part of 1960. These microporous membranes were constructed out of cellulose acetate/nitrate and were primarily for general use in the laboratory. Large scale applications of membrane processes became possible with the advent of the asymmetric membrane. [Loeb. 1962]. These were initially used in the desalination industry. One of the most recent innovations has been the development of a 'third' generation membrane manufactured

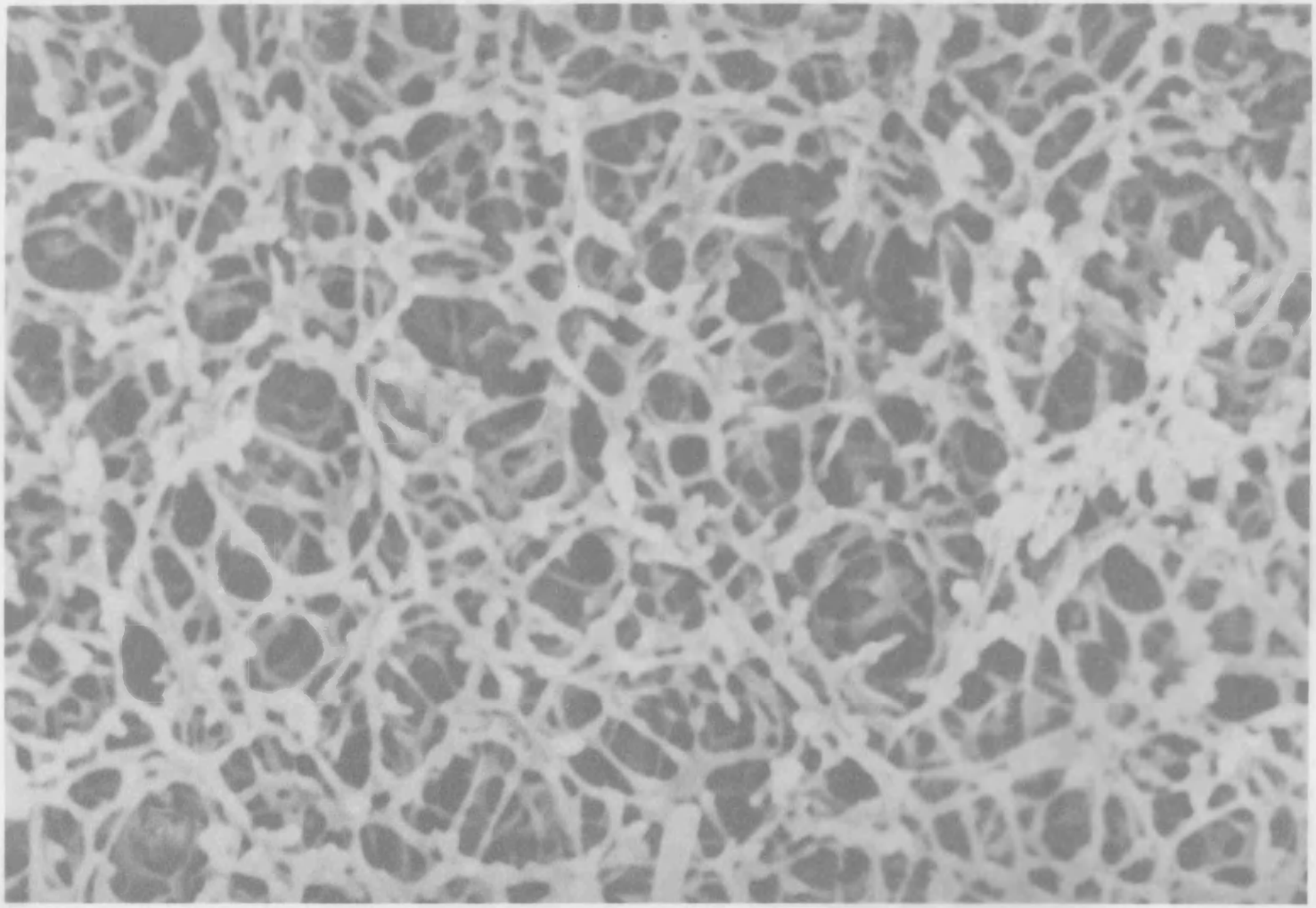
from a composite base material. This product has found widespread use in the pervaporation industry for the dehydration of organic solvents.

MEMBRANE MANUFACTURE

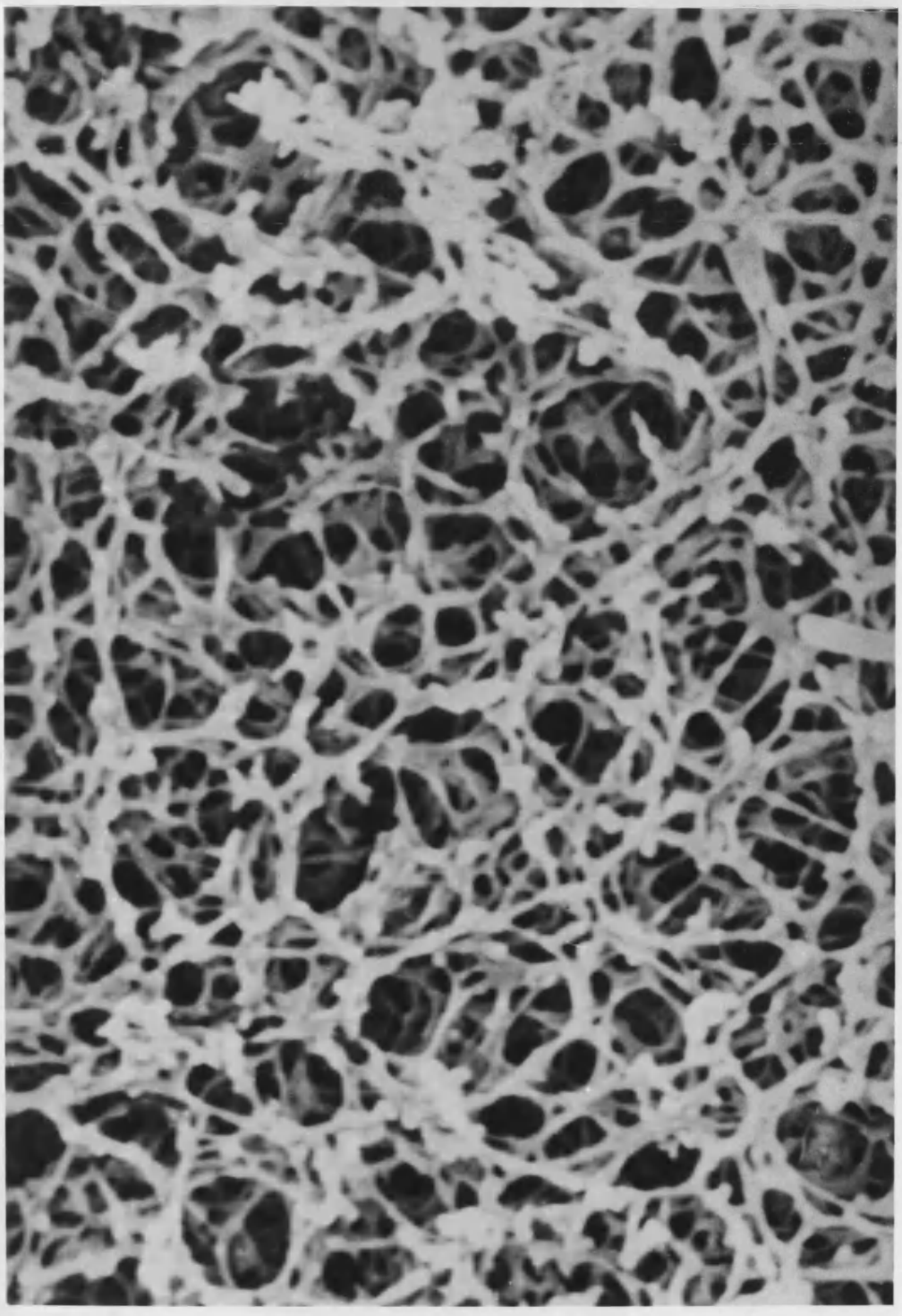
Two basic approaches are generally used in the manufacture of microfiltration polymeric membranes. In both the wet and dry phase inversion process, the polymer, which is dissolved in a solvent/non-solvent system, is poured onto a flat surface. Evaporation of the solvent is then allowed to take place, under carefully controlled conditions of temperature, air flow and humidity. This event begins to have a pronounced effect on the solubility of the polymer. At this point the initially homogenous colloidal suspension known as the *sol* is converted into the *gel*. In the wet process complete solvation is prevented by immersing the polymer film in a quench bath, to remove the remaining solvents and pore forming agents. The dry process allows complete evaporation of the solvent, no quench stage is used.

A typical scanning electron micrograph of an asymmetric cellulose microfiltration membrane is illustrated in Fig 4. The high degree of asymmetry is believed to impart better flow properties to the structure.

Figure 4



Cellulose microfiltration membrane. (X 5,000)



CHAPTER II : PREPARATION OF A METAL CHELATE AND A p-ABA BIOSELECTIVE MEMBRANE ADSORBENT

The aim of this investigation was to develop a novel affinity adsorbent in the form of a membrane. This had to be achieved according to the following criteria, namely, the membranes had to be relatively inexpensive to produce, convenient to handle and they had to be capable of processing biological fluids both quickly and effectively. The experimental procedures used to prepare these affinants are outlined in the opening section of this chapter, followed by a discussion and evaluation of the results.

SECTION 2.1 - MATERIALS AND METHODS

2.11 - MATERIALS

Cellulose membranes, 4Å Asypor^{R} microfiltration membranes (Domnick Hunter Filters Ltd)
dioxane (BDH), CDI (Sigma), sodium hydroxide (Aldrich), hydrochloric acid (Aldrich) sodium carbonate (Sigma), hexanoic acid (Sigma), MES (Sigma), p-ABA (Sigma), copper sulphate (Aldrich), 1,4—butanedioldiglycidyl ether (Aldrich, containing 95% ether), sodium borohydride (Sigma), sodium hydroxide (Sigma), IDA (Sigma), zinc chloride (Sigma), sodium phosphate (BDH),

pH monitor (Alpha 500, Oxford Laboratories), incubation shaker (New Brunswick Scientific), spectrophotometer (CE 588 Cecil Instruments)

METHODS

PREPARATION OF THE p-ABA CELLULOSE MEMBRANES

2.12 - ACTIVATION OF CELLULOSE MEMBRANES WITH CARBONYLDIIMIDAZOLE

Three cellulose membranes (150 mg dry wt.) were pre-soaked in distilled water and then solvent exchanged into dioxane (50 ml). This material was left to equilibrate for 24 hrs after which time dioxane (15ml) containing CDI (1mMol) was added. This mixture was shaken for 2 hrs. The final product was washed with dry solvent (200ml) and stored at 4^{o}C in a solution of sodium azide.

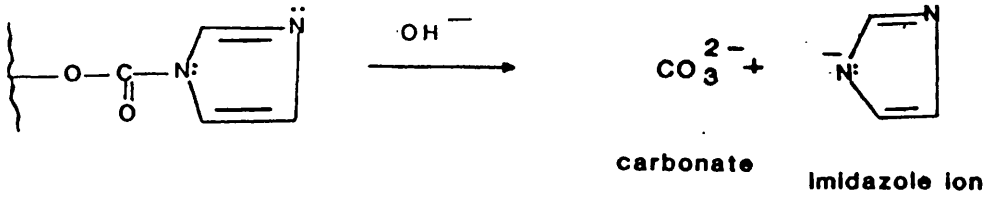
ASSAY PROCEDURE [Bethell 1979]

The CDI matrix (150 mg dry wt.) was transferred to a solution of sodium hydroxide (50 ml, 0.2M carbonate free) and left to hydrolyse for 1 hr. A 25ml aliquot of this solution was titrated against hydrochloric acid (0.1M) under nitrogen, between pH 9 and pH 4. After blowing off the carbon dioxide produced during the titration

procedure, the pH of the medium was adjusted back to pH 9 by the addition of sodium hydroxide (0.1M). The base was then retitrated down to pH 4.

The first titration indicated the total amount of carbonate and imidazole present. The second procedure gave the total imidazole content of the matrix, in a 25ml sample of hydrolysate. (Scheme 1) (See also appendix 2).

SCHEME 1



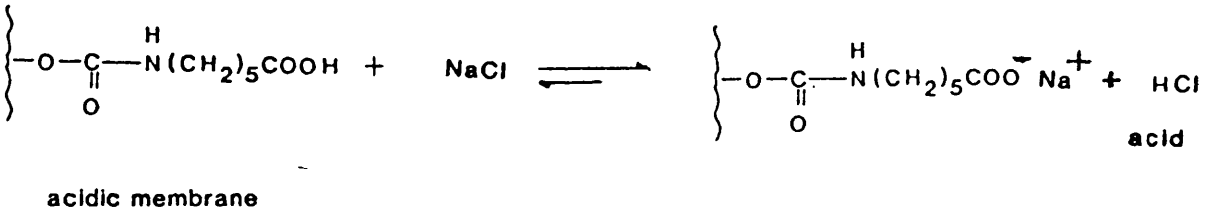
2.13 - COUPLING OF 6-AMINOHEXANOIC ACID TO THE CDI MATRIX

The CDI activated matrix was added to a solution of sodium carbonate/sodium bicarbonate (6 ml, pH10) containing hexanoic acid (3.4 mMol), and left to shake at room temperature for 16 hrs. The product was finally washed with hydrochloric acid (250ml, 0.02M), sodium hydroxide (250ml, 0.02M) and distilled water (500ml), and stored at $4^{o}C$ until required.

ASSAY PROCEDURE [Bethell 1981]

The activated material was cut and placed into an aqueous solution of sodium chloride (10ml, 1M). This mixture was titrated against NaOH (0.1M), to neutrality. (Scheme 2) (See also appendix 2).

SCHEME 2



acidic membrane

2.14 - COUPLING OF p-AMINOBENZAMIDINE TO THE SPACER MOLECULE

The hexanoic acid derivative (150 mg) was placed in a solution of MES. (20 ml, 0.2M, pH4.75) containing EDC (1M). This suspension was allowed to shake at room temperature for 30min. p-Aminobenzamidine (0.18 mMol) was then added to the mixture, which was then shaken for an additional 16 hrs. During the first 60 min of this reaction, the pH of the medium dropped. This required the careful addition of NaOH (0.1M) to maintain the pH at 4.75. The product was finally washed with hydrochloric acid (100ml, 0.05M), sodium hydroxide (100ml, 0.05M) and distilled water (250ml), and stored at $4^{o}C$ in sodium azide solution.

ASSAY PROCEDURE [Bethell 1981]

The washings from the reaction mixture were combined, and the volume adjusted to 1 litre with sodium dihydrogen phosphate (0.1M, pH7). The adsorbance of this solution was measured at 292 nm (molar extinction coefficient$_{292}$ = 1.53 x 10^4 mol^{-1} $cm^{-1}L$).

2.15 - PREPARATION OF THE METAL CHELATE CELLULOSE MEMBRANES

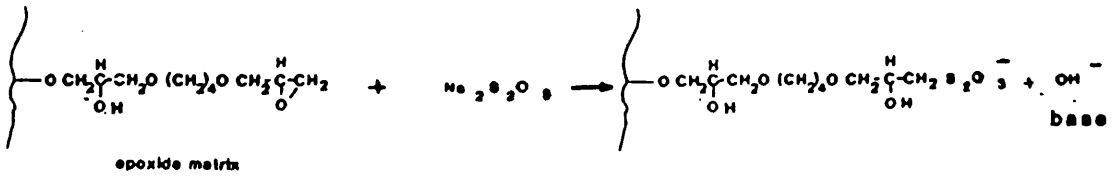
ACTIVATION OF THE CELLULOSE MEMBRANES WITH 1,4 - BUTANEDIOLDIGLYCIDYL ETHER (BDDE)

Two membranes (200mg dry wt.) were allowed to shake for 16 hrs with a suspension of BDDE (1ml) in aqueous sodium hydroxide (5ml, 0.3M) containing sodium borohydride (1mg/ml). The final product was washed with distilled water (500ml) and stored at 4^{o}C in a solution of sodium azide.

ASSAY PROCEDURE [Porath 1975]

The activated membranes were cut and placed into a solution of sodium thiosulphate (5ml, 1.3M). The liberated base was titrated to neutrality with hydrochloric acid (0.1M). (Scheme 3) (See also appendix 2).

SCHEME 3



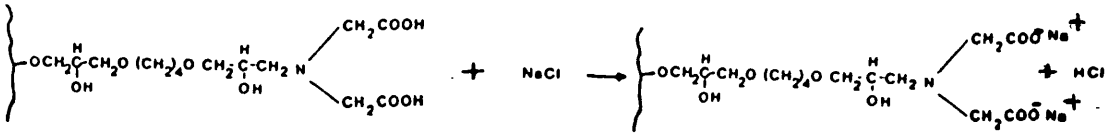
2.16 - COUPLING OF THE METAL CHELATE LIGAND AND ADDITION OF THE METAL ION

The epoxide membranes (200mg dry wt.) were transferred to a solution of sodium carbonate (5ml, 2M) containing iminodiacetic acid (0.2g/ml) and left to shake at 65^{o}C for 12 hrs. The reaction product was washed with distilled water (500ml) and inserted into a Sartorius filter holder. A solution of zinc chloride or copper sulphate (1mg/ml) was passed through the membrane unit, at a flow rate of 1 ml/min, until the acid chelate was saturated.

ASSAY PROCEDURE

The acid derivatives were placed into a solution of sodium chloride (10ml, 1M) and titrated against sodium hydroxide (0.1M) until neutrality. (Scheme 4) (See also appendix 2).

SCHEME 4



chelate membrane

SECTION 2.2 INTRODUCTION TO CDI ACTIVATION

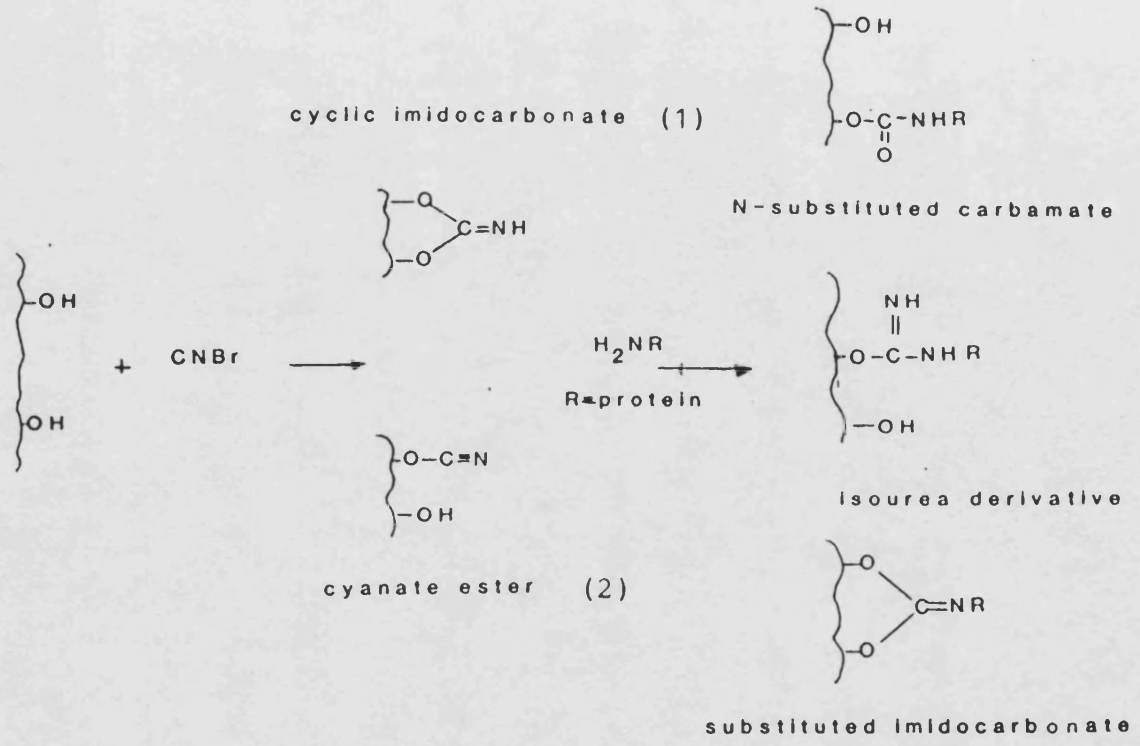
The past decade has witnessed the introduction of multiple of novel affinity techniques employing a numerous array of adsorbent materials. It was surprising therefore, to find one method in particular dominating the area of matrix activation. I refer here to CNBr derivatisation.

The reaction of CNBr with hydrophilic polysaccharides was first documented by Patty [1949] as a method for the technological processing of cellulose. It was originally introduced into the field of affinity chromatography through the work of Axen [1967]. Since those early investigations the precise chemistry of the activation procedure has been under constant review. [Bartling 1972, Kennedy 1980]. From these studies the following conclusions have been made. The treatment of cellulose with CNBr produces two products, a cyclic imidocarbonate (1) and a cyanate ester (2). These intermediates can then proceed to covalently couple an $-NH_2$ containing molecule such as a protein or peptide in one of three possible ways. (Scheme 5).

This protocol however has two severe disadvantages. Sevensson [1973], using isoelectric focussing, found that the main product formed from the coupling reaction was the basic isourea derivative. These groups impart undesirable anion exchange properties to the matrix. Secondly, the products of coupling are extremely susceptible to ligand leakage. Tesser [1974] prepared a range of CNBr derivatised adenosine adsorbents and concluded that the leakage of affinant took place predominantly from the site of formation on the surface of the solid carrier.

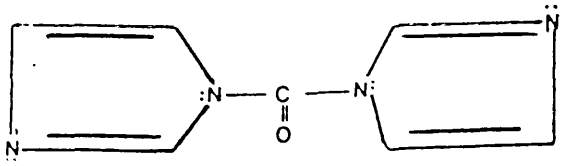
As a consequence of these problems a vigorous search for alternatives to the CNBr procedure quickly ensued.

SCHEME 5


cyclic imidocarbonate (1)
N-substituted carbamate
CNBr
H_2NR
R=protein
isourea derivative
cyanate ester (2)
substituted imidocarbonate

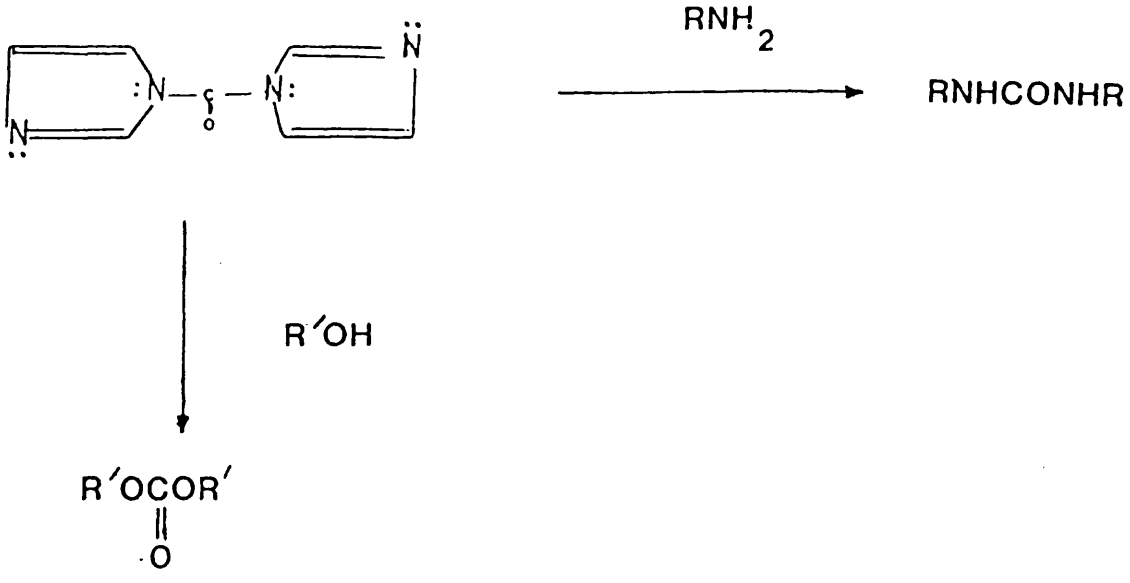
In response to this quest Bethell [1979] developed an ingenious activation synthesis based on the chemical reactivity of N,N-carbonyldiimidazole; (CDI), a two ring heterocycle (Fig 5).

FIGURE 5



This molecule is capable of undergoing relatively facile nucleophilic substitution reactions with both alcohols and amines forming the respective carbonates and ureas [Staab 1957], (Scheme 6).

SCHEME 6



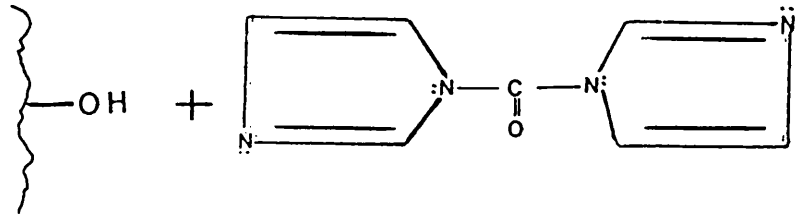
Similarly the synthesis of small polypeptides has been achieved through acylation of the imidazoyl ring, [Anderson 1960]. The results of subsequent CDI investigations [Bethell. 1981, 1981b, 1981c] were extremely encouraging. Ultimately one could obtain exceptionally high activation levels under relatively mild conditions, and most importantly the surface of the matrix is devoid of charged groups. In addition the CDI compound is a relatively inexpensive material which is commercially readily

available. In light of the apparent simplicity of the activation process it was decided to adopt this technique to activate the cellulose membranes.

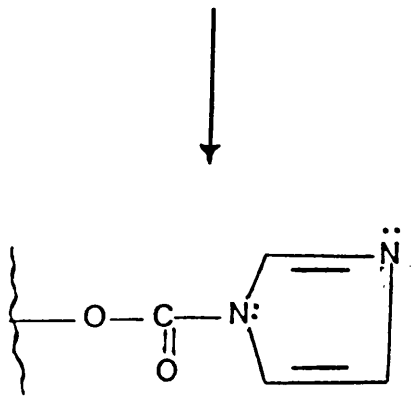
CARBONYLDIIMIDAZOLE CELLULOSE MEMBRANE ACTIVATION

The activation of the cellulose matrix with CDI proceeds smoothly to generate the imidazoyl carbamate analog [Scheme 7]. (See methods section 2.12).

SCHEME 7



matrix CDI



activated matrix

The carbonylating reagent is extremely susceptible to hydrolysis in aqueous solution, hence the reaction was carried out in dioxane previously dried over sodium wire. One of the major advantages of this method of activation is the formation of an exceptionally stable urethane link between the support and the CDI molecule. Between the pH range 8-10, 15 hours was required to completely hydrolyse the matrix. Rogers [1986] compared the stability of a TSK 4000 PW^{R} resin after CNBr

activation to the same resin activated with CDI. He found that although the CNBr material initially had a greater capacity to bind β-lactamase, this was considerably reduced after a relatively short time. In contrast the CDI matrix had a much slower binding capacity decay over the same period.

The range of substitution levels one obtains is strongly dependant on the initial CDI concentration. [Table 2] (refer also to fig 6).

TABLE 2

CDI ADDED [mMoles]	ACTIVATION LEVEL [mMoles]*
0.25	0.12
0.5	0.26
1	0.34
2	0.43
3	0.47
6	0.51

* (using 150 mg dry wt. of cellulose membrane)

At high levels of activation there is a tendency to generate bridging cyclic carbonate groups across the pores of the material [Fig 7]. These groups are quite unreactive and effectively reduce the number of available activation sites [Hearn, 1986].

Figure 6

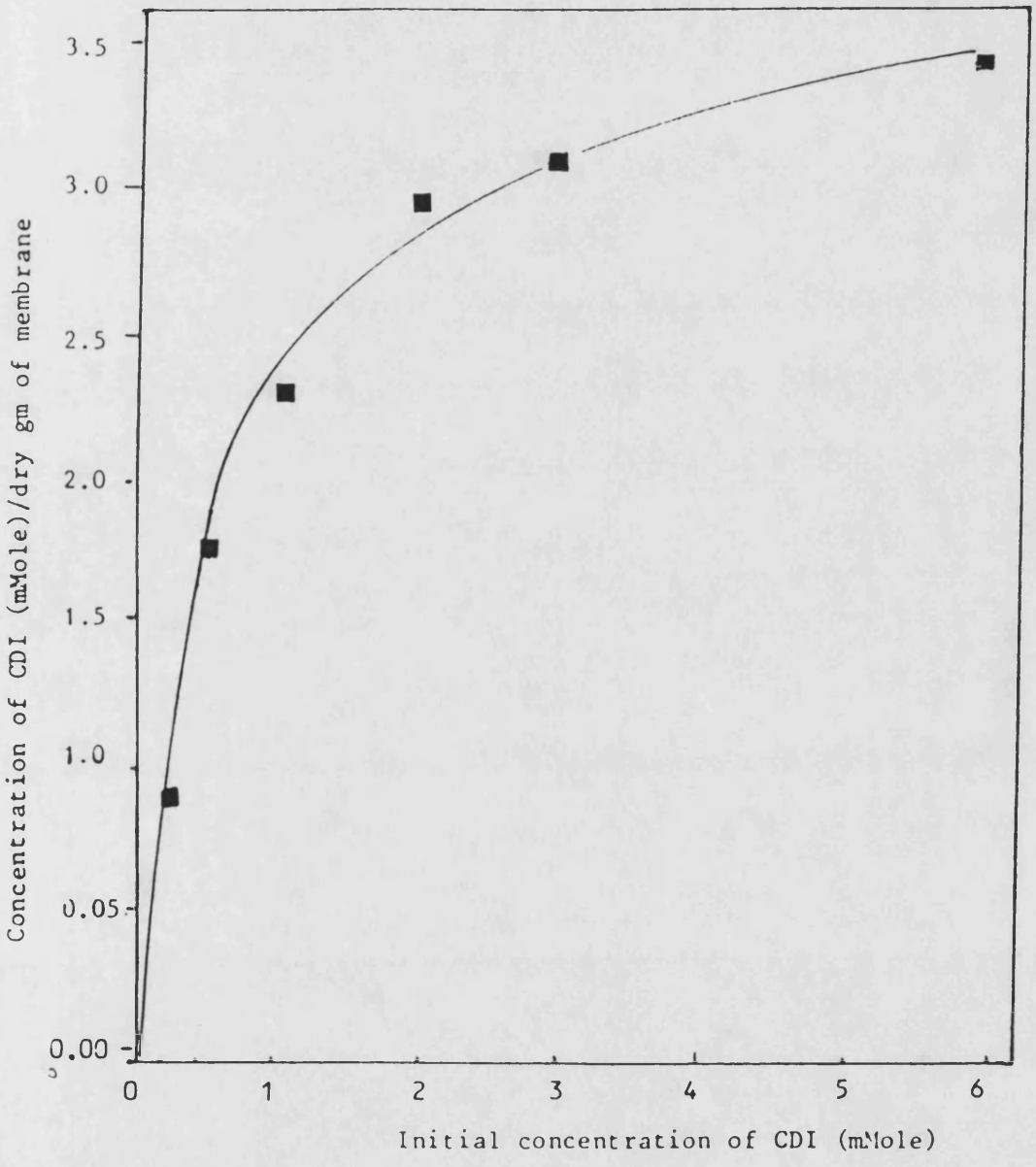
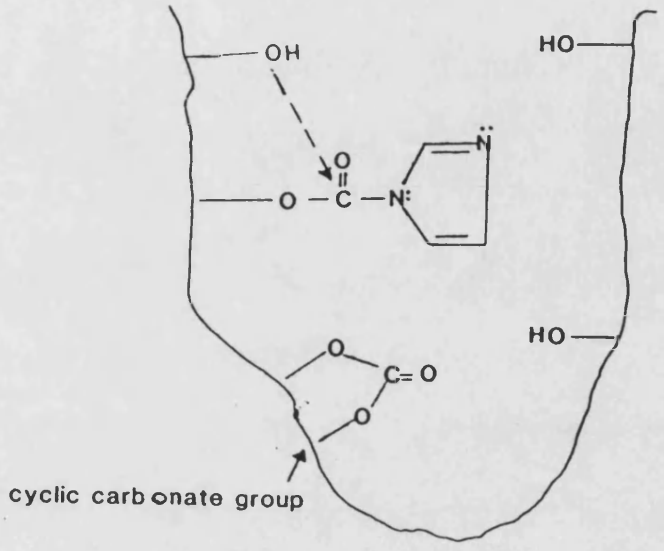

3.5
3.0
2.5
2.0
1.5
1.0
0.05
0.00
0
1
2
3
4
5
6
Concentration of CDI (mMole)/dry gm of membrane
Initial concentration of CDI (mMole)

FIGURE 7



The activated species is relatively stable to oxygen nucleophiles and can therefore be handled quite readily in aqueous solution. It will however undergo N-nucleophilic substitution reactions.

SECTION 2.3 COUPLING REACTION OF 6-AMINOHEXANOIC ACID (AHA)

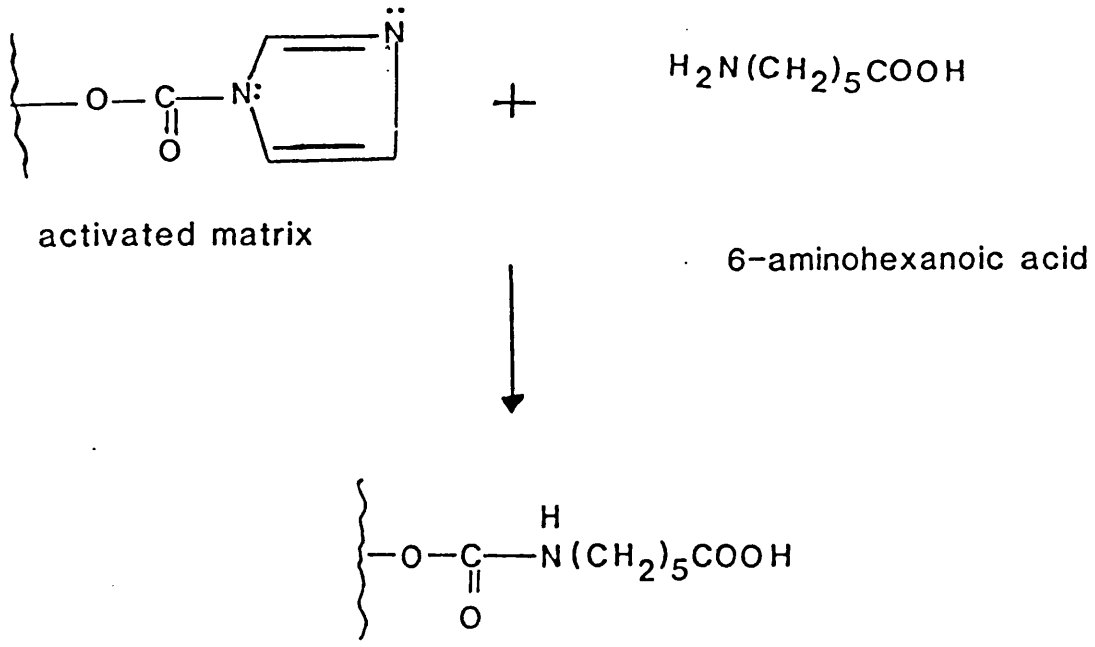
When the ligand to be immobilised has a molecular weight in excess of 5K daltons a spacer molecule is generally interposed between the matrix and bioligand to improve its steric accessibility for the macromolecule [Cuatrecasas, 1970, 1971b]. The ligand is usually a linear hydrocarbon of varying chain lengths. The length is a critical parameter. If the molecule is to small it is rendered virtually ineffective, conversely if it is too large one may introduce the unwanted problem of hydrophobicity into the system [Lowe. 1973].

The importance of interposing a hydrocarbon chain between the ligand and matrix backbone was illustrated by the relative ineffectiveness of Sepharose - D-tryptophan methyl ester compared to the hexanoic acid ester, to bind α-chymotrypsin [Cuatrecasas 1968].

There are basically two approaches used to couple a spacer group to the matrix. [Barry. 1973, 1974]. In the first method the bioligand-chain complex is synthesised in solution and then coupled to the support, alternatively the leash is first bound onto the matrix onto which the bioligand is coupled in a second step. The latter method is recognised as being the more straightforward of the two procedures.

The second protocol was used to couple 6-aminohexanoic acid to the CDI activated membrane, to generate the corresponding acid-carbamate derivative [scheme 8]. (see methods section 2.13).

SCHEME 8



It has been shown that an amino leash couples best at a pH value within one unit of its pKa [Bethell [1981]]. Hence the reaction was carried out at pH 10. (pKa of hexanoic acid = 11).

The concentration of COOH groups on the surface of the membrane was measured as a function of the initial hexanoic acid concentration used in the reaction process. [Table 3] (refer also to fig 8 and methods section 2.13).

TABLE 3

AHA ADDED [mMoles]	ACTIVATION LEVEL [mMoles]*
0.34	0.018
0.52	0.023
0.87	0.037
1.73	0.052
3.4	0.100
6.8	0.100

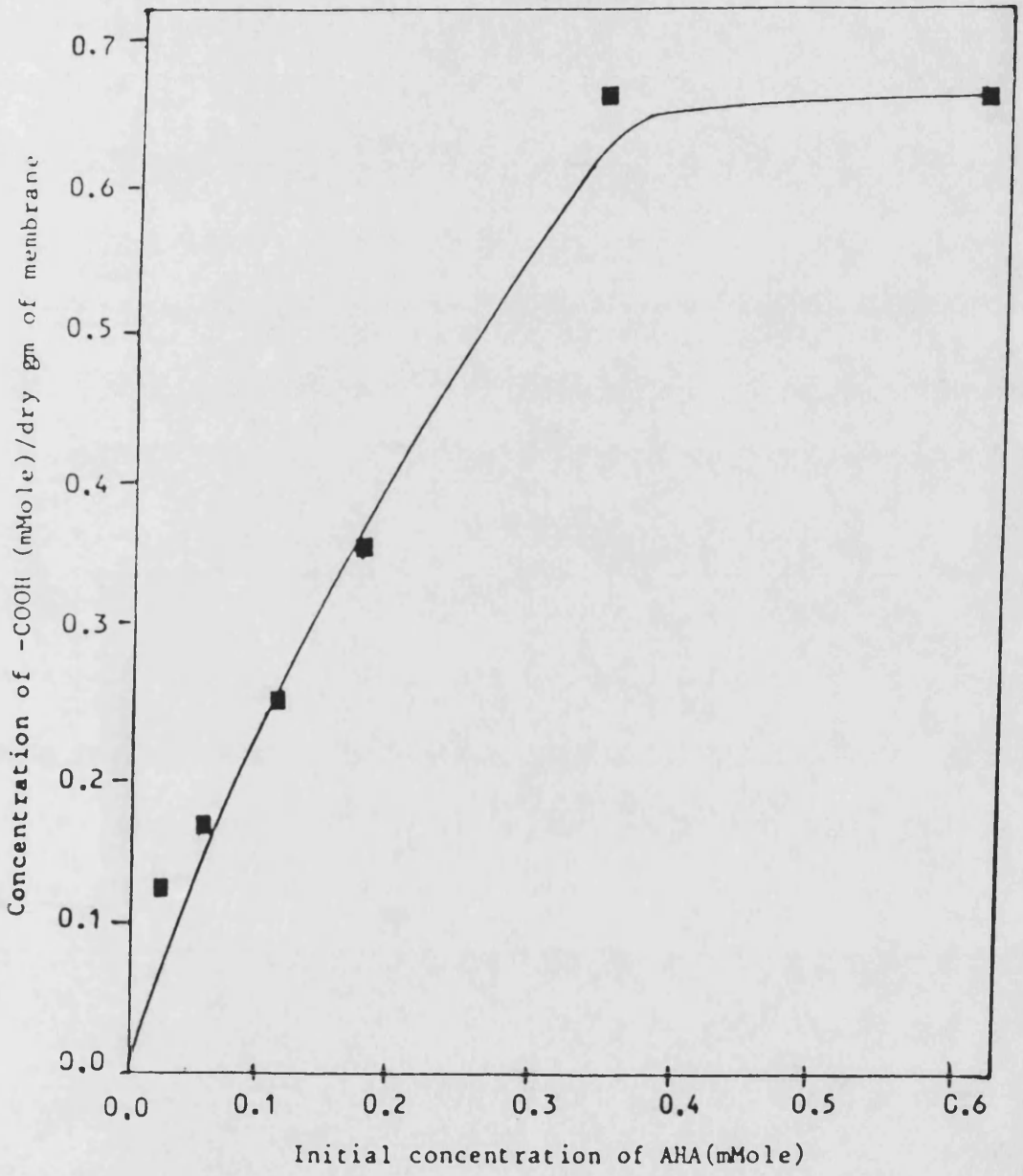
* (previously activated with 1mMol CDI)

The acid membrane could now be used in a subsequent step to couple the bioligand of one's choice.

SECTION 2.4 : ATTACHMENT OF p-AMINOBENZAMIDINE : A SPECIFIC SERINE PROTEASE INHIBITOR

During the mid-1960's Mares-Guia [1965] performed a series of kinetic studies on a family of derivatives, that had the ability to inhibit the protease activity of trypsin and chymotrypsin; p-ABA was one member of this family. Hixon [1973] later coupled this molecule to a hexanoic spacer group using the condensation reagent EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride). This compound is

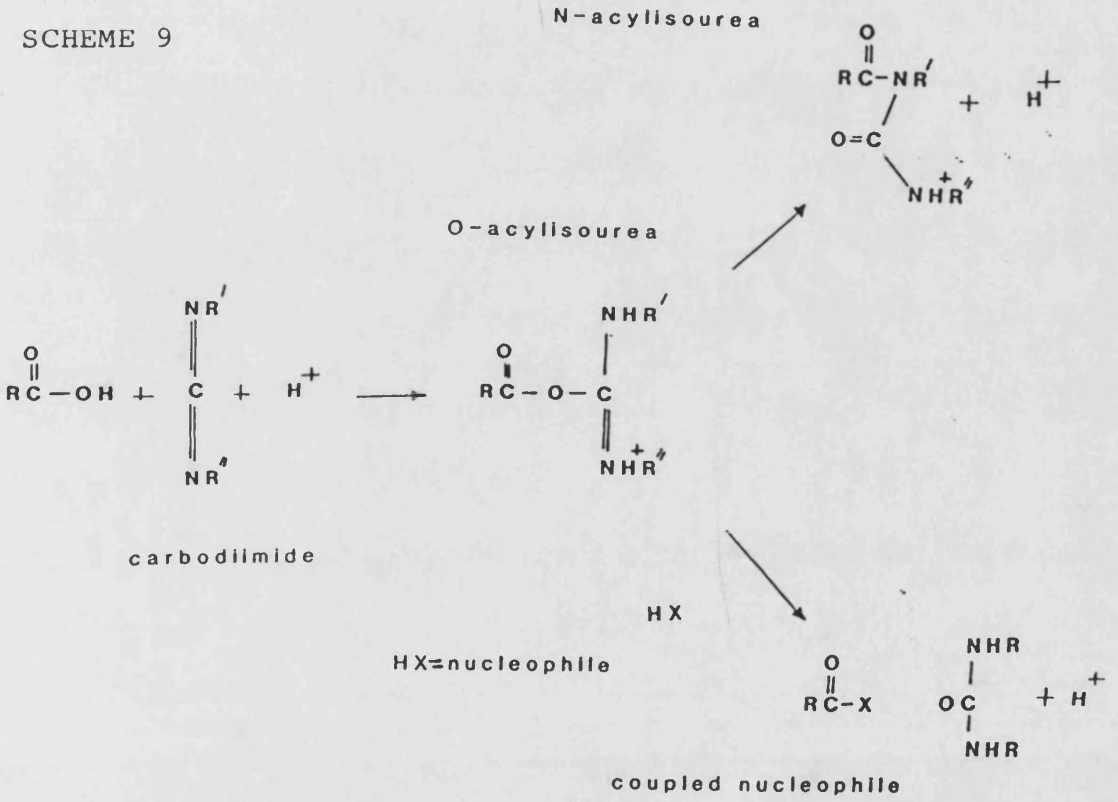
Figure 8


Concentration of -COOH (mMole)/dry gm of membrane
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Initial concentration of AHA(mMole)

one of a number of soluble carbodiimides that are used extensively to promote the synthesis of small polypeptides [Tengblad. 1981, Lyon 1981]

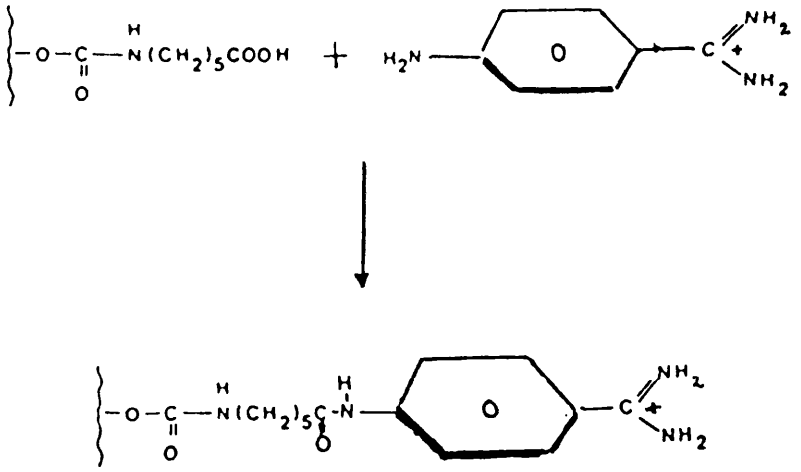
Hoare [1967] has shown that the reaction of EDC with a carboxylic acid is initiated by the addition of the carboxyl group across one of the diimide double bonds to generate the O-acyl urea. This product can then further react in one of two ways. It can either rearrange to produce the N-acyl urea, or it can undergo nucleophilic substitution. [Scheme 9].

SCHEME 9



p-Aminobenzamidine is nucleophilic and can inhibit the intramolecular rearrangement reaction, alternatively forming the N-substituted derivative (scheme 10).

SCHEME 10



The concentration of p-ABA on the membrane was measured as a function of the initial EDC molarity. [Table 4] (refer also to fig 9 and methods section 2.14).

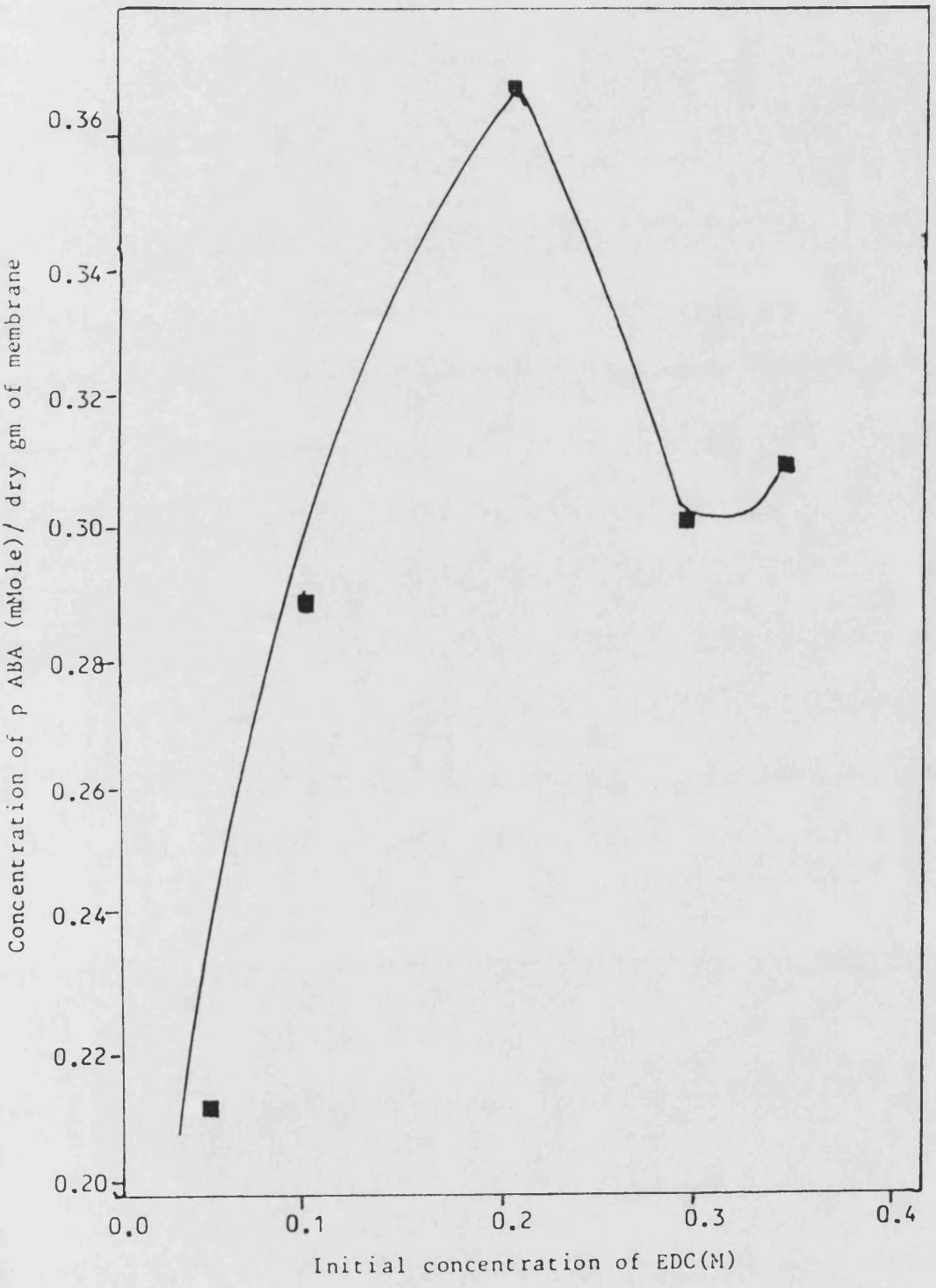
TABLE 4

EDC [M]	ACTIVATION LEVEL [mMoles]*
0.05	0.032
0.1	0.044
0.2	0.055
0.3	0.046
0.35	0.047

* (previously treated with 3.4 mMoles AHA)

The functionalised membranes could be stored at $4^{o}C$ in a solution of azide for several months without any apparent loss of activity. The ability of this material to selectively adsorb serine proteases will be discussed in Chapter IV.

Figure 9

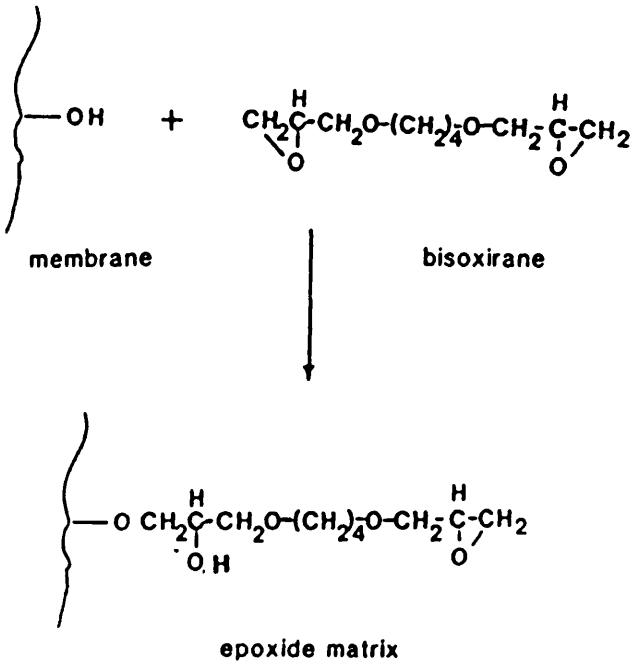

0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
Concentration of p ABA (mMole)/ dry gm of membrane
0.0
0.1
0.2
0.3
0.4
Initial concentration of EDC(M)

SECTION 2.5 : PREPARATION OF A METAL CHELATE MEMBRANE

Porath [1974] successfully introduced reactive oxirane groups onto the surface of agarose beads using a class of bifunctional compounds known as bisoxiranes. Bisoxiranes are long chain, hydrophilic, non-charged molecules, which contain within their structure two reactive epoxide rings. These functional groups can undergo base catalysed S_N2 reactions with a variety of Lewis bases, especially hydroxyl containing molecules such as polysaccharides. This is a highly desirable reaction in affinity chromatography, for it not only forms an exceptionally stable ether link between the matrix and the bioligand, but, as a consequence of the length of the molecule, it removes the necessity of attaching a spacer ligand in a second step. These reagents are also capable of cross-linking a polysaccharide to enhance its mechanical stability.

The reaction of 1,4-butanedioldiglycidyl ether with the cellulose membranes proceeds according to scheme 11.

SCHEME 11



There was a substantial decrease in the free oxirane content of the matrix when the concentration of base was increased above 0.3M [Table 5] (refer also to fig 10 and

methods section 2.15). This could be attributed to an increased tendency of the reagent to cross-link the membrane under highly basic conditions. This effect is negated if higher concentrations of epoxide are used. [Table 6] (refer also to fig 11 and methods section 2.15).

TABLE 5

NaOH [M]*	EPOXIDE [γ moles]
0.1	5
0.2	12.5
0.3	17.5
0.5	6
1	-

(* In 10% epoxide solution using 200mg dry wt. of cellulose membrane)

TABLE 6

EPOXIDE [%v/v]*	EPOXIDE [γ moles]
10	17.5
20	26
30	47

(*In 0.3M base using 200mg dry wt. of cellulose membrane)

This species can now readily undergo nucleophilic substitution with a variety of chelating agents. The most commonly used chelating ligand is iminodiacetic acid: IDA. [Porath. 1975, 1981]. This reacts quite smoothly with the oxirane membrane to give the diacid derivative, that has the ability to retain strongly metal ions [Scheme 12].

Figure 10

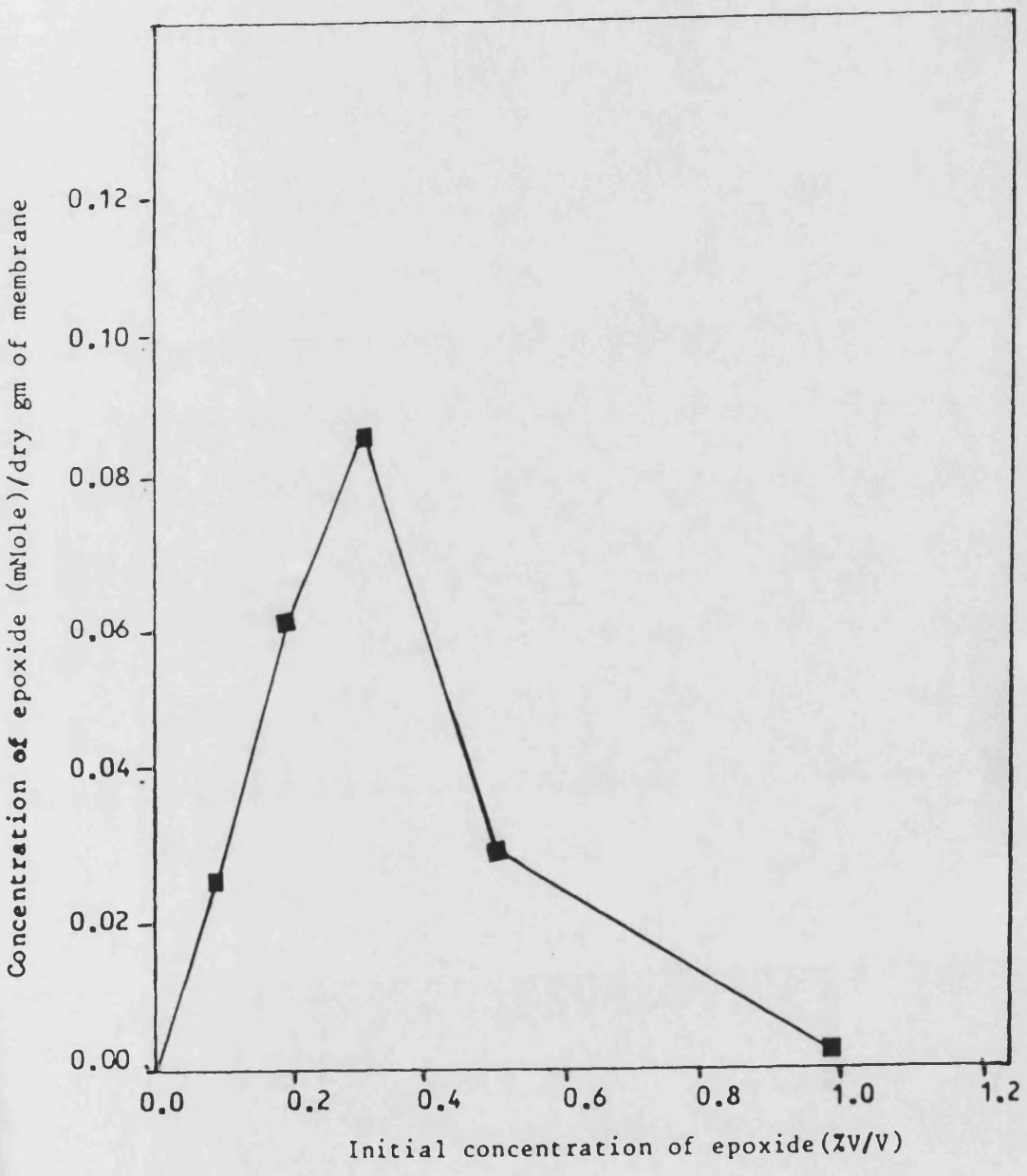
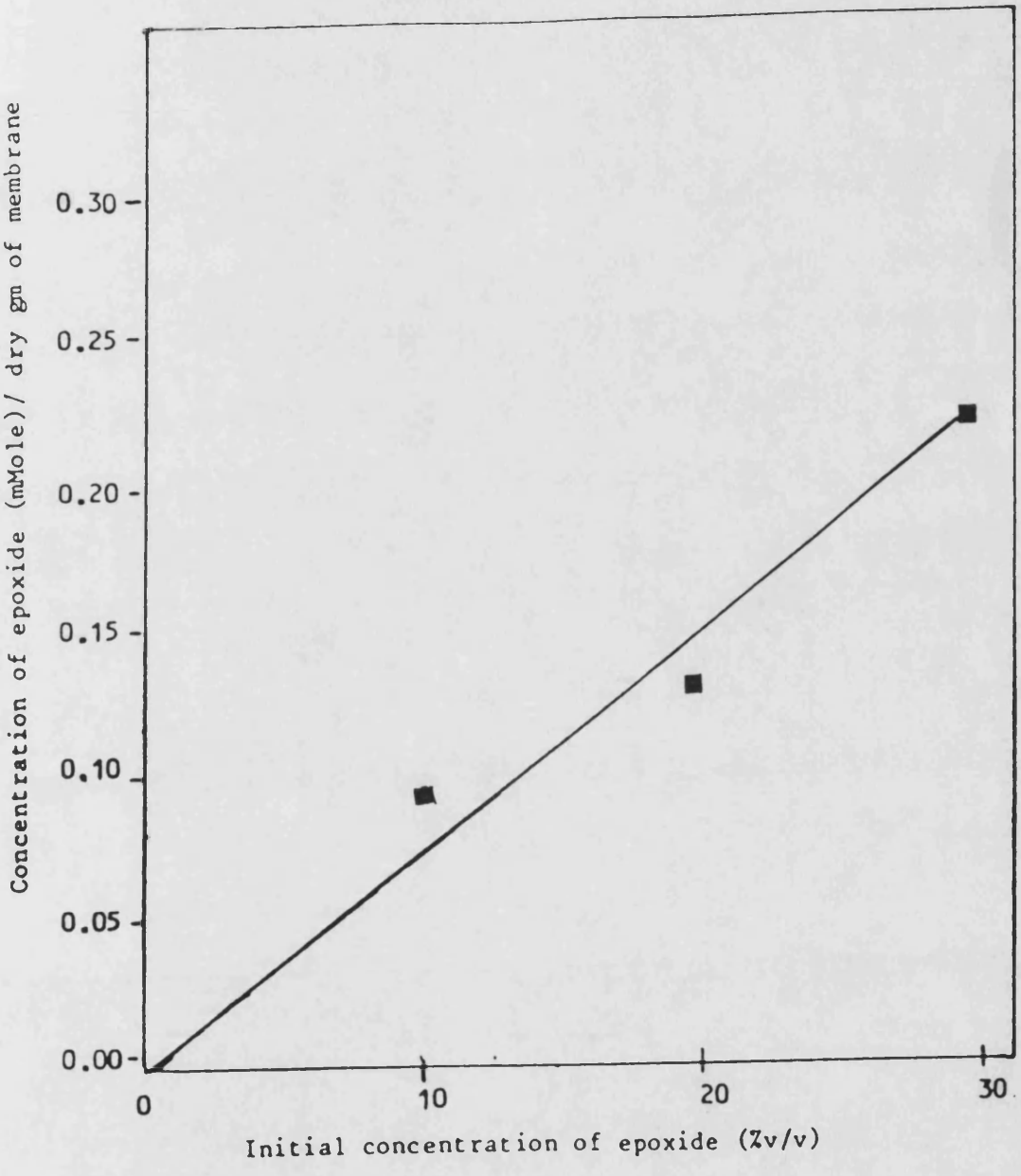
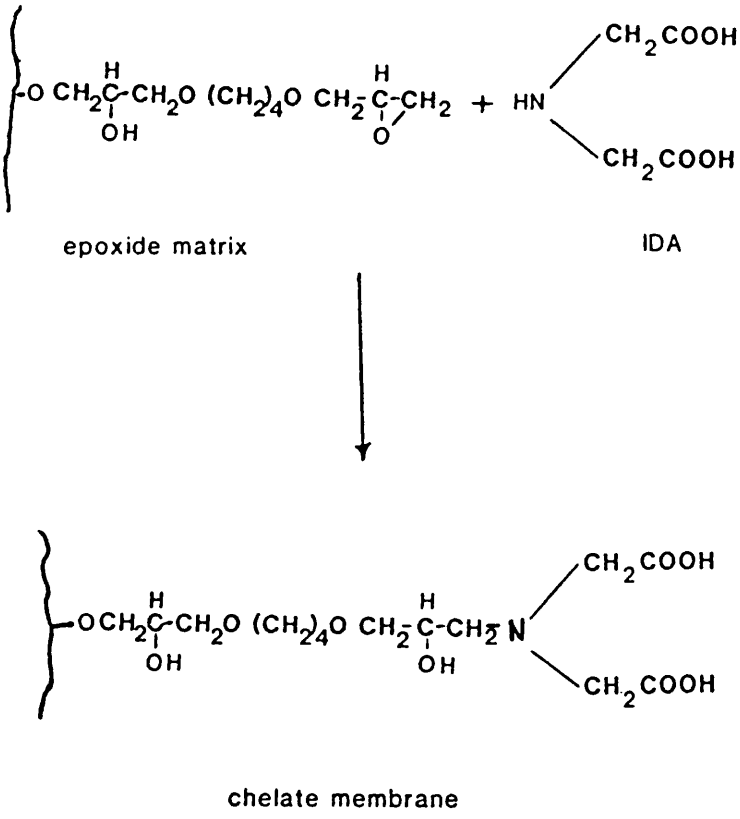

Concentration of epoxide (mMole)/dry gm of membrane
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Initial concentration of epoxide (%V/V)

Figure 11


Concentration of epoxide (mMole)/ dry gm of membrane
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
Initial concentration of epoxide (%v/v)

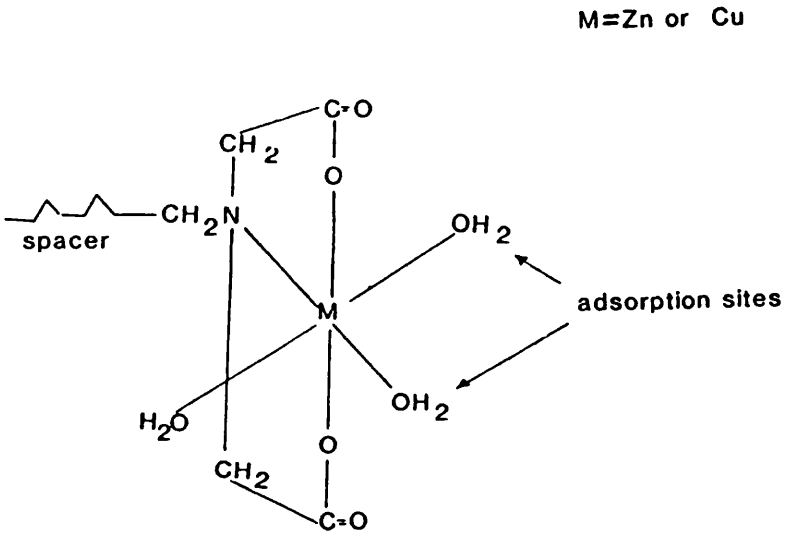
SCHEME 12



Metal ions are highly solvated in aqueous solution as a result of coordination of water molecules. This water of solvation can be replaced by a stronger base resulting in a metal complex. The base is usually a molecule containing nitrogen, sulphur and/or oxygen atoms. IDA has two donor oxygen atoms and a nitrogen atom in its structure, and is therefore called a tridentate chelate. The binding of a metal to this ligand is much stronger than one would find for a simple monodentate complex.

The metal was introduced into the arms of the chelate by passing a solution of its salt through the membrane matrix. The possible orientations of the ligands around the central atom is illustrated in Fig 12, (refer also to methods section 2.16).

FIGURE 12



Unlike the p-ABA species these membranes are less specific in binding protein. This will however be discussed in section 4.2 together with their applications.

CHAPTER III : QUANTITATIVE AFFINITY CHROMATOGRAPHY

This chapter attempts to quantify the phenomena of affinity adsorption using a simple model to describe the binding performance of the p-ABA membranes prepared in section 2.2. An interactive computer simulation package was used to determine the kinetics of the batch adsorption of trypsin onto these membranes. The influence of temperature and pH on the kinetics of the binding reaction was also ascertained.

SECTION 3.1 MATERIALS AND METHODS

3.11 - MATERIALS

Trypsin (Sigma), sodium phosphate (BDH), sodium chloride (Aldrich), spectrophotometer (CE 500, Cecil Instruments), shaking water bath.

3.12 - METHODS

The kinetics of the batch adsorption experiments were monitored using the following procedure. For each batch study, a known concentration of trypsin (0.125 mg/ml - 1mg/ml) in phosphate buffer (50ml, 0.25M, pH8 (7)) containing sodium chloride (0.25M), was added to a fixed volume of p-aminobenzamidine activated cellulose membranes (7.07 cm^3) in a temperature controlled water shaking bath. At specific time intervals, a sample (1ml) of the soluble phase was removed from the batch reactor and placed into a u.v. spectrophotometer which measured the resultant protein concentrations.

This process was repeated until an equilibrium had been established between the solid and mobile phases.

The K_{de} and q_m values obtained from these experiments together with the values for C_o, M, V, and tfin, (for explanation of these parameters see nomenclature) were inserted into an interactive computer simulation package called ISIM. This programme numerically integrates the rate expression given in eqn 8 (see programme listing and section 3.2) to generate a series of concentration values as a function of time. The programme listing is given below.

ISIM PROGRAMME LISTING

```
:MODEL DESCRIPTION
  CONSTANT KD =1.2000E-5,QM=1.50E-4,C0=3.30E-5,M=7.07, V=50,
  CONSTANT CINT=10,K1=225
     INITIAL                                        tfin=1000
       t=0;Q=0
DYNAMIC
  Q'=K1*((QM-Q)*(C0-M/V*Q)-KD*Q)
  C=C0-M/V*Q
C1=C/C0
OUTPUT T,C1
PREPARE T,Q,C1
$ VAL KD       =  1.20e-05
$ VAL V        =   50.000
$ VAL K1       =   225.00
```

After specifying the experimental variables, an estimated value of k_1 is introduced into the rate expression, which the programme then uses to construct a theoretical time-course profile of the binding reaction. Several values of k_1 were used. The actual value of k_1 was taken to be that which most accurately described the experimental data.

SECTION 3.2 : INTRODUCTION TO QUANTITATIVE AFFINITY CHROMATOGRAPHY

The rapid growth in quantitative affinity chromatography began just over a decade ago [Graves 1974, Wankat 1974]. A range of theoretical approaches have been specifically developed to describe the biochemical principles on which it is based. In order to advance these basic concepts, one must firstly understand the complex physical and chemical nature inherent to biological interactions. The phenomena of biological recognition and the consequent formation of a reversible, non-covalent complex, primarily occurs through a combination of electrostatic, hydrophobic, Van der Waals and London dispersion forces.

The formation of an enzyme-bioligand complex at a single site is generally described by the equilibrium given in Eqn. 1.

$$\underset{\text{Enzyme}}{[E]} + \underset{\text{Ligand}}{[L]} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{\text{Complex}}{[EL]} \qquad \text{Eqn. 1}$$

The process is more suitably expressed in terms of the dissociation constant K_d [Eqn 2].

$$K_d = \frac{[E][L]}{[E\ L]} = \frac{k_2}{k_1} \qquad \text{Eqn. 2}$$

K_d is a parameter that characterises the strength of affinity between the enzyme and bioligand. Enzyme-substrate interactions for example, tend to be relatively labile and exhibit K_d values of the order of $< 10^{-5}$M. In contrast antibody-antigen binding is exceptionally strong with dissociation constants commonly of the order 10^{-8}M-10^{-14}M.

Chase [1983] characterised the K_d value in terms of the concentration of available binding sites on the adsorbate [Eqn 3].

$$K_d = \frac{c^* b^*}{q^*} \qquad \text{Eqn. 3}$$

* = Equilibrium value

c = concentration of adsorbate in solution (moles/cm^3)

b = concentration of vacant binding sites (moles/cm^3)

q = concentration of adsorbate bound (moles/cm^3)

There was however one problem with this relationship, namely, in practice not all the available binding sites are accessible. The immobilised ligand can be orientated on the matrix in such a manner that makes it sterically inaccessible to the adsorbate. Furthermore, bound ligand can exert a shielding effect on neighbouring binding sites, precluding their interaction with additional bioligand, [Hixon, 1973]. Kasche [1982] has reported that in some affinity systems less than 0.1% of the available binding sites were utilised. In situations where these phenomena prevail, the actual K_d value could be much higher than the K_d determined from solution experiments.

To overcome this drawback Chase [1983] modified eqn. 3 to express an effective dissociation constant: K_{de} [Eqn 4].

$$K_{de} = \frac{c^*(q_m - q^*)}{q^*} \qquad \text{Eqn. 4}$$

q_m = Theoretical maximum amount of adsorbate bound (moles/cm^3).

This equation can be rearranged to give an expression for q [Eqn 5].

$$q^* = \frac{q_m c^*}{(K_{de} + c^*)} \qquad \text{Eqn. 5}$$

Equation 5 predicts that the adsorption isotherm should be non-linear and of the type first described by Langmuir [1916], often termed a favourable isotherm.

SECTION 3.3: BATCH ADSORPTION ANALYSIS OF TRYPSIN BINDING ONTO p-ABA ACTIVATED CELLULOSE MEMBRANES

The adsorption of trypsin using a p-ABA membrane cartridge has previously been characterised and reported [Carter and Howell, see appendix 1]. As an extension of this work, a series of predicted adsorption isotherms have been obtained using a simple mass-balance relationship to describe the adsorption-desorption process.

The isotherms were constructed by applying different concentrations of trypsin solution to a constant volume of adsorbent, from which an equilibrium value for each of the respective protein concentrations was ascertained. [Table 7] (refer also to methods section 3.12). The non-linearity of the isotherm [Fig 13] was consistent with the model proposed by Chase [1983]. The linearised form has an intercept at $-K_{de}$ and a slope of $1/q_m$ [Fig 14]. The K_{de} value of this adsorption reaction is marginally higher than the value of 8.25 x 10^{-6}_{M} obtained by Mares-Guia [1965] from his studies on trypsin binding in free solution.

TABLE 7

Trypsin Conc. Co mg/ml	C^* ($\times 10^{-8}$ moles cm^{-3})	q^* ($\times 10^{-7}$ moles cm^{-3})	$\frac{C^*}{q^*}$
0.125	0.228	0.207	0.110
0.250	0.428	0.436	0.098
0.500	1.040	0.737	0.141
1.000	2.600	1.11	0.236

This might be explained in terms of the rates of adsorption and desorption which are not simply described by the respective forward and backward rate constants k_1 and k_2, but will also include significant contributions from resistances to mass transfer. Fook [1982] from his protein adsorption studies on ion-exchange cellulose concluded that the initial period of the adsorption process was controlled by film diffusion. Rather than rigorously introducing these effects into an overall mass-balance, Chase [1984] used an approximation, and assumed that the rate of adsorption could be described by a total rate constant k_1. [Eqn 6].

$$\frac{dq}{dt} = k_1 c^* (q_m - q) - k_2 q = 0 \text{ at equilibrium} \qquad \text{Eqn 6}$$

For a batch system of initial adsorbate concentration c_o, volume of adsorbent v total volume of adsorbate V; the concentration of unbound adsorbate given by Eqn 7.

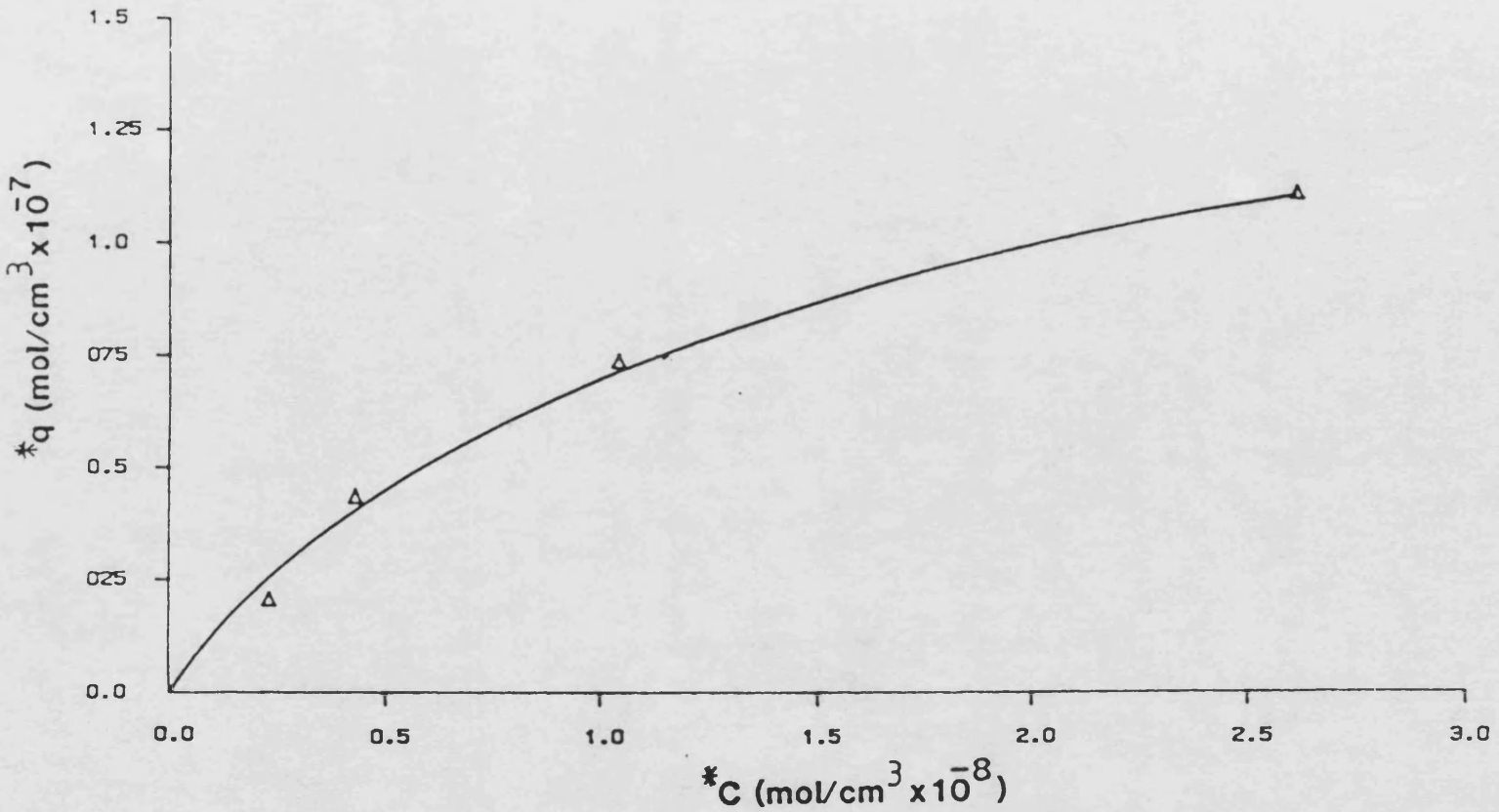
$$c^* = \frac{c_o - vq}{V} \qquad \text{Eqn 7}$$

Substituting this expression into Eqn 6.

$$\frac{dq}{dt} = k_1 \left(\left(c_o - \frac{vq}{V} \right) (q_m - q) - K_d q \right) \qquad \text{Eqn 8}$$

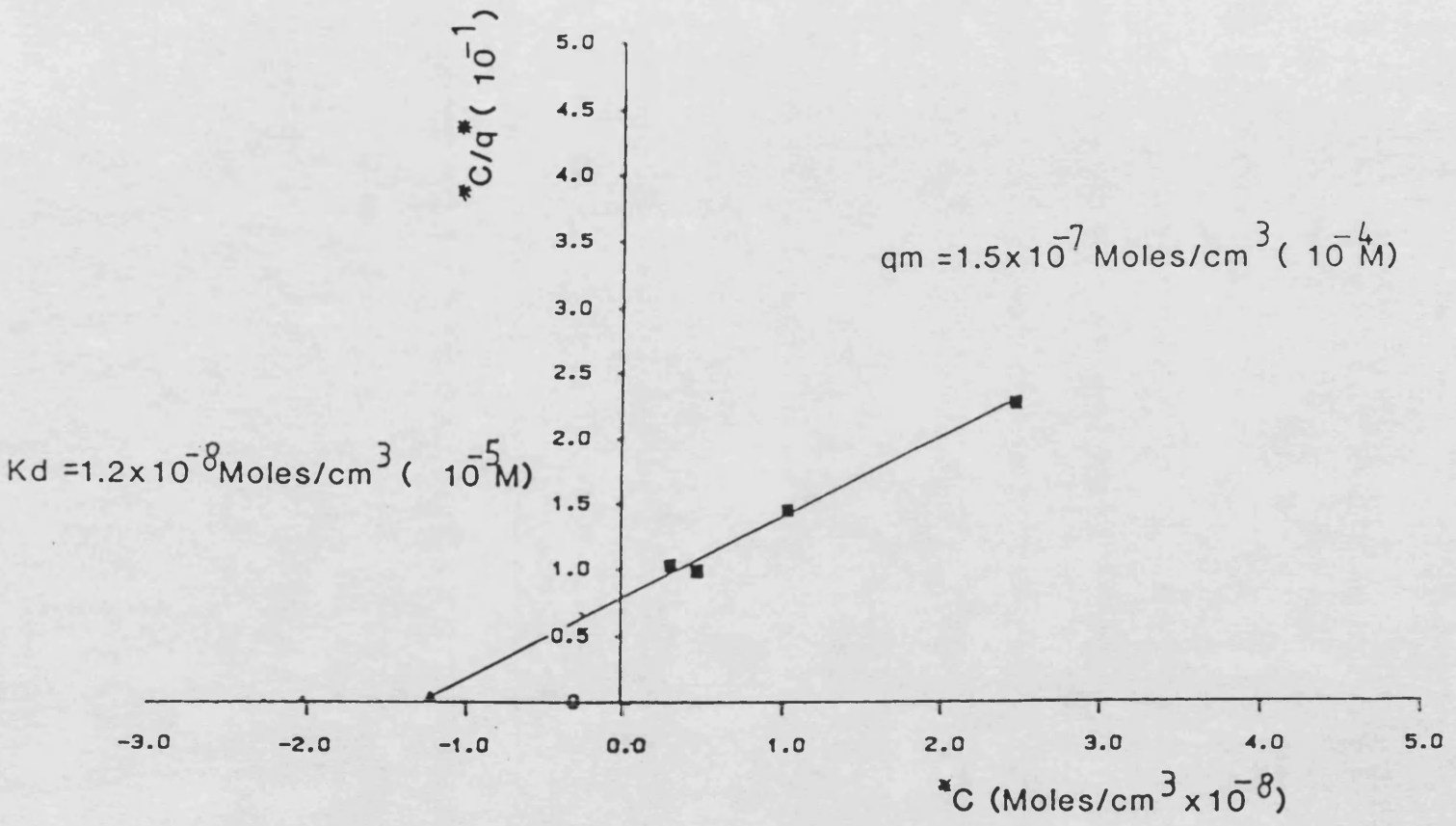
A series of predicted adsorption time-course profiles were constructed by computer simulation using Eqn 8 (see methods section 3.12) in conjunction with the values of K_{de} and q_m obtained from the batch analysis experiments [Fig 14]. The k_1 value for the adsorption process was considered to be that which most suitably represented the experimental data. Although the k_1 values varied slightly between different protein concentrations the theoretical and experimental components of this

Figure 13



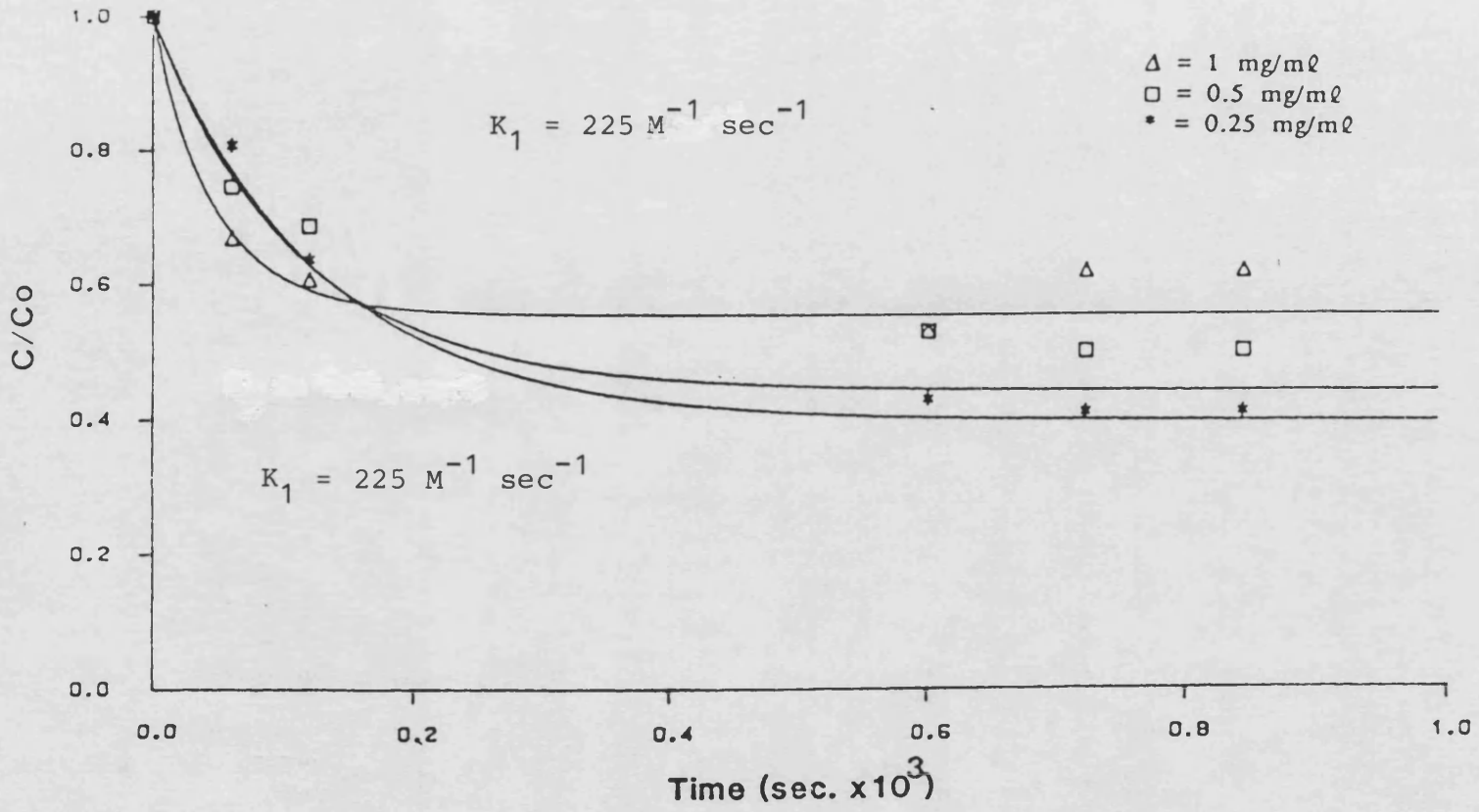
Langmuir adsorption isotherm for the binding of trypsin onto p-ABA activated cellulose membranes. pH8, 20°C.

Figure 14



Linearised Langmuir adsorption isotherm for the binding of trypsin onto p-ABA cellulose membranes pH8, 20°C

Figure 15



Time-monitored adsorption of trypsin onto p-ABA activated cellulose membranes. pH8, 20°C
(Theoretical = – , Experimental = symbol)

investigation are in fact in reasonable agreement, which suggests that the total mass-balance is adequately described by Eqn 8. [Fig. 15]

To check the validity of these results a useful expression first derived by Graves [1974], was modified by Chase [1983] to describe the proportion of total adsorbate that would be bound at equilibrium (q^*_p), when a volume v of adsorbent is added to a volume V of crude material containing adsorbate initially at a concentration c_o [Eqn 9].

$$q^*_p = \frac{vq_m}{2c_oV} \cdot (F) \left(1 - \sqrt{1 - \frac{4c_oV}{F^2 q_m v}} \right) \qquad \text{Eqn} \quad 9$$

$$\text{where} \quad F = \frac{K_{de}(V + v)}{q_m v} + \frac{c_oV + 1}{q_m v}$$

Table 8 lists a series of observed and predicted results

TABLE 8

c_o mg/ml	PREDICTED (%)	OBSERVED (%)
0.125	56	56
0.250	53	59
0.500	46	50
1.000	34	37

Once again there is a reasonable agreement between the experimental data and theoretical predictions.

SECTION 3.4 THE EFFECT OF TEMPERATURE ON THE KINETICS OF THE BATCH ADSORPTION

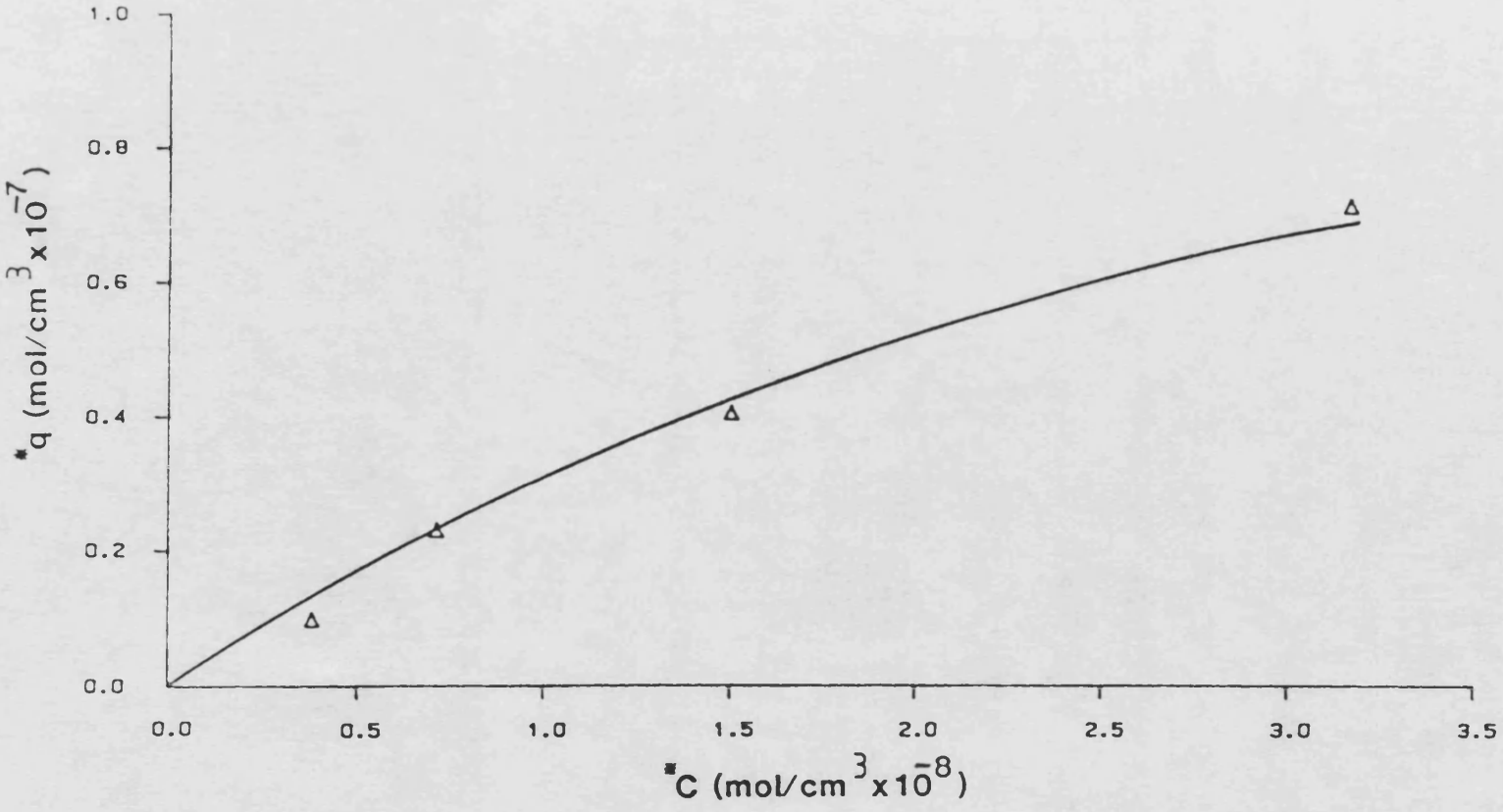
Adsorption of a dissolved substance from the mobile phase onto the stationary phase is generally an exothermic process. Usually, the more exothermic the reaction, the more sensitive it will be to any changes in temperature. If the adsorption process is accompanied by a decrease in ethalpy i.e. evolution of heat ($-\Delta H$) increasing the temperature of the system should lead to a decrease in the amount of adsorption.

This temperature dependence can be utilised to separate enzymes with different enthalpies of binding. Lowe [1974] for example, found that the ability of N^6-(6-aminohexyl)5-AMP-Sepharose to bind B.S.A. decreased substantially upon increasing the temperature. This effect was also expressed in the p-ABA-trypsin interaction, in which the adsorption process became less favourable as the temperature was raised. This conclusion was drawn from the observation of a five-fold increase in the value of K_{de} [Figs 16,17] [Table 9].

TABLE 9

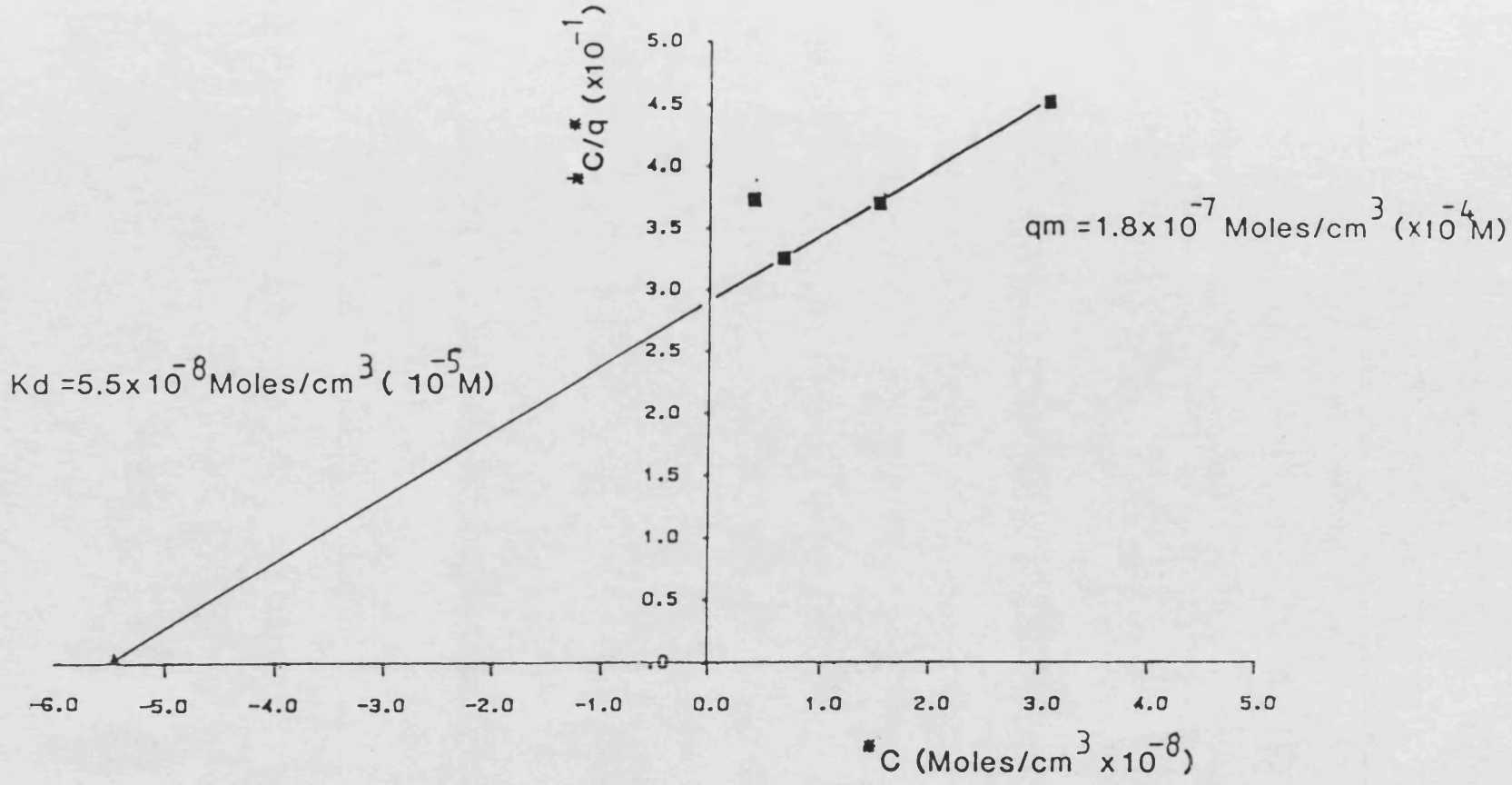
Trypsin Conc. Co mg/ml	C^* ($\times 10^{-8}$ moles cm^{-3})	q^* ($\times 10^{-8}$ moles cm^{-3})	$\frac{C^*}{q^*}$
0.125	0.38	0.98	0.38
0.250	0.71	2.30	0.32
0.500	1.50	4.04	0.37
1.000	3.16	7.11	0.44

Figure 16



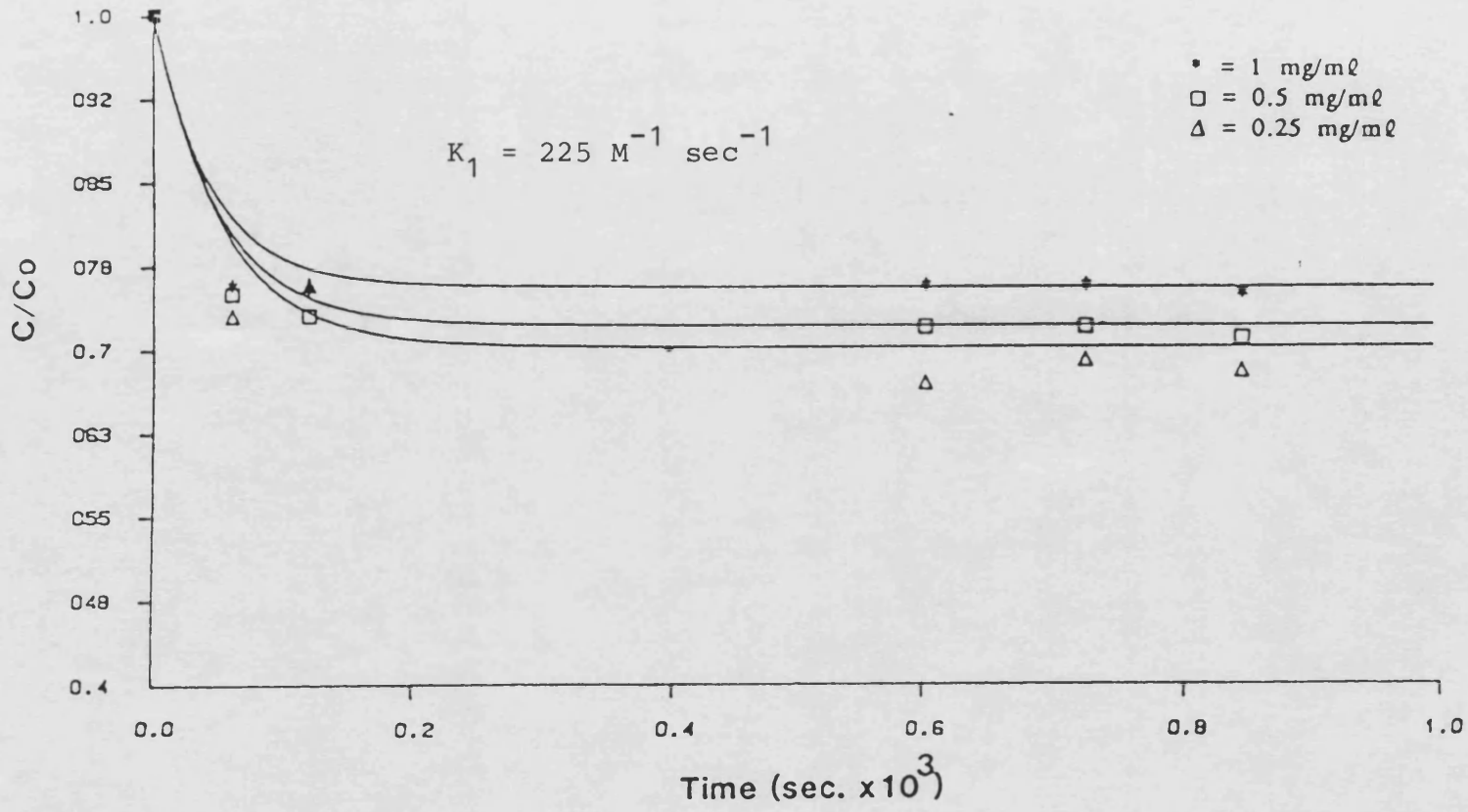
Langmuir adsorption isotherm for the binding of trypsin onto p-ABA activated cellulose membranes. pH8, 30°C.

Figure 17



Linearised Langmuir adsorption isotherm for the binding of trypsin onto p–ABA cellulose membranes pH8, 30 °C

Figure 18



Time-monitored adsorption of trypsin onto p-ABA activated cellulose membranes. pH8, 30°C
(Theoretical = – , Experimental = symbol)

The experimental data was subjected to the same time-course prediction analysis performed in section 3.2 [Fig 18]. From this study one important conclusion emerges. There was an approximately 40% reduction in the amount of protein bound by the matrix for each of the respective trypsin concentrations.

SECTION 3.5 THE EFFECT OF pH ON THE KINETICS OF THE BATCH ADSORPTION

The catalytic effect of enzymes is usually limited to within a narrow pH range of the optimal pH, at which both the enzyme and its substrate are ionised. A shift from the optimal pH results in a decrease in either the rate of enzymatic reaction or the affinity of the system for the substrate or both.

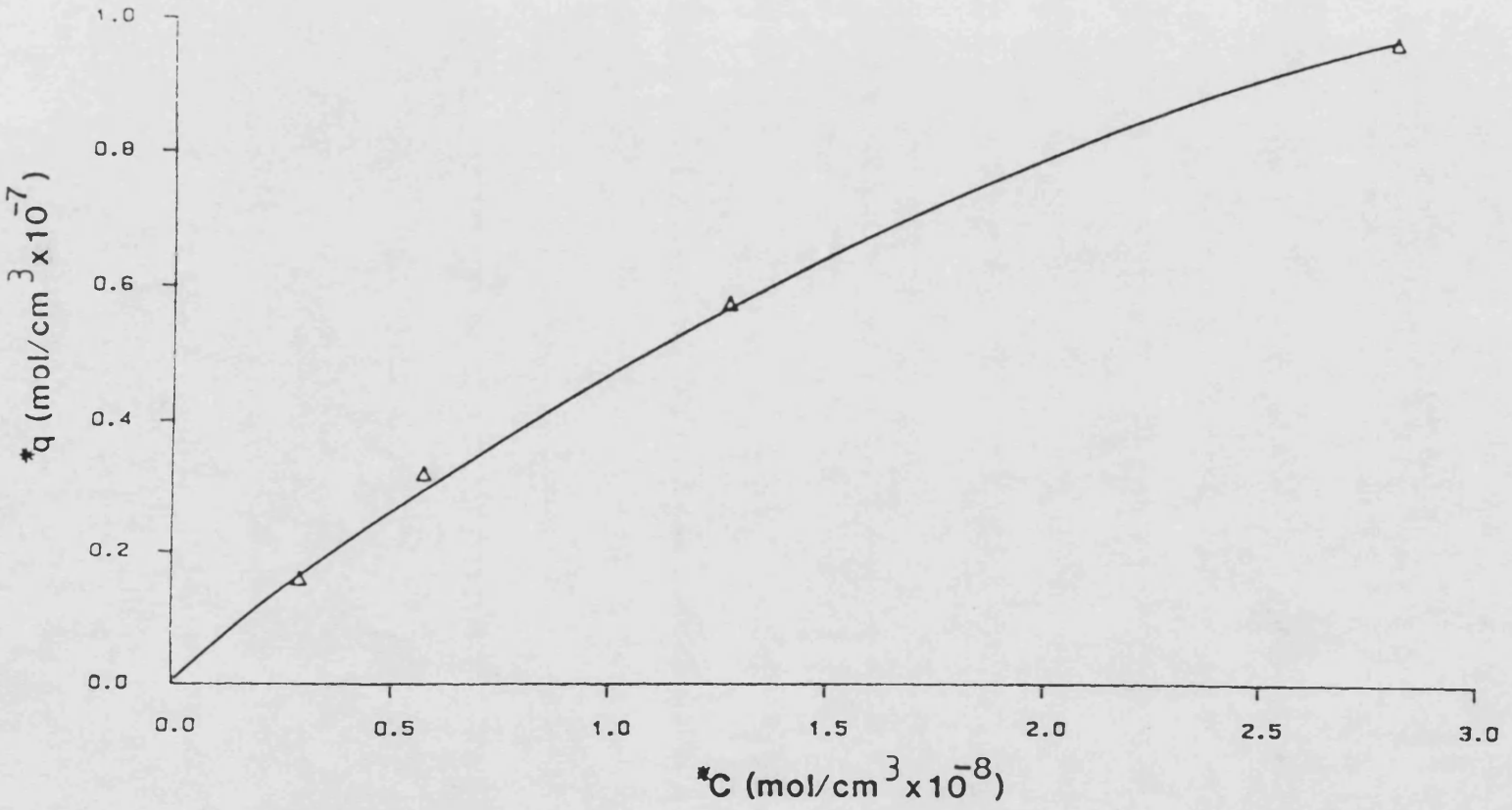
Trypsin is known to undergo optimal binding under basic conditions. This event has previously been monitored at pH8. Decreasing the basiscity to pH7 produced a three-fold increase in the value of K_{de} [Figs 19,20,21] [Table 10].

TABLE 10

Trypsin Conc. Co mg/ml	C^* ($\times 10^{-8}$ moles cm^{-3})	q^* ($\times 10^{-8}$ moles cm^{-3})	$\frac{C^*}{q^*}$
0.125	0.29	1.59	0.182
0.250	0.57	3.20	0.178
0.500	1.27	5.72	0.220
1.000	2.80	9.60	0.290

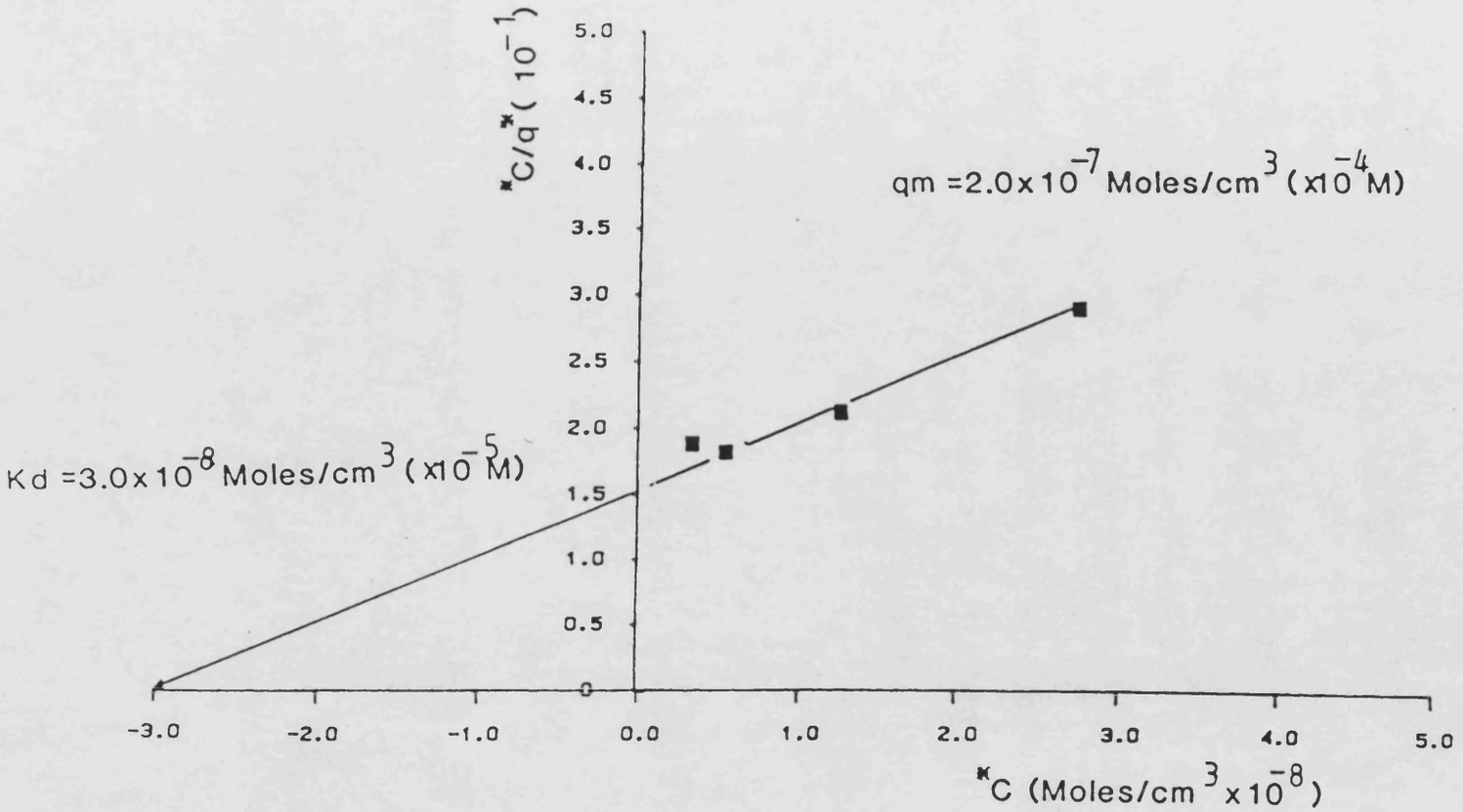
It therefore becomes apparent from these investigations, that the success or failure of affinity adsorption system will essentially be influenced by the initial operating conditions.

Figure 19



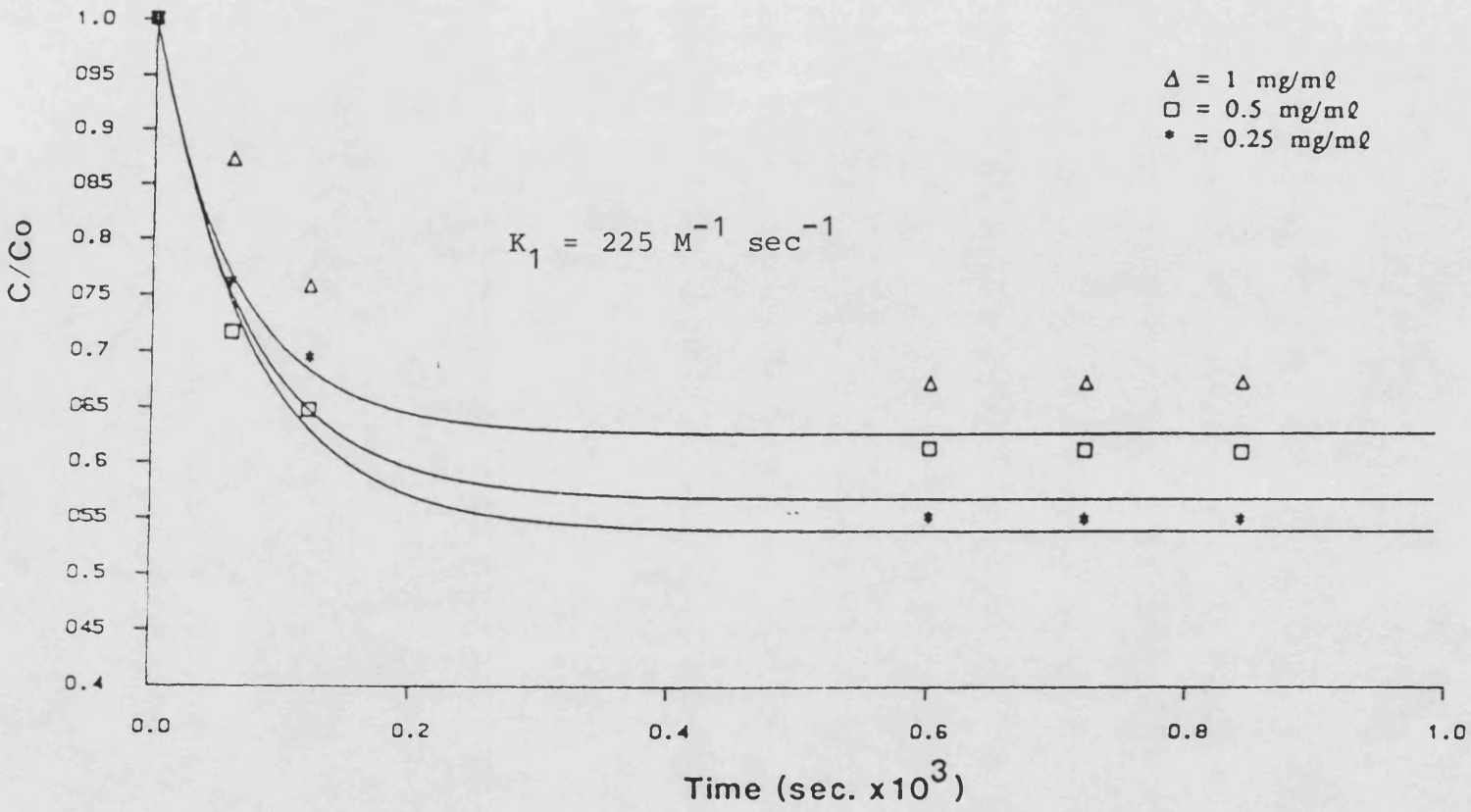
Langmuir adsorption isotherm for the binding of trypsin onto p-ABA activated cellulose membranes. pH7, 20°C.

Figure 20



Linearised Langmuir adsorption isotherm for the binding of trypsin onto p-ABA cellulose membranes pH7, 20°C

Figure 21



Time-monitored adsorption of trypsin onto p-ABA activated cellulose membranes. pH7, 20°C
(Theoretical = - , Experimental = symbol)

CHAPTER IV: PROTEIN FRACTIONATION : METAL CHELATE CHROMATOGRAPHY

During the final stages of this investigation the metal chelate and p-ABA membrane derivatives were subjected to a series of separation studies, to determine their effectiveness as an affinity adsorbent. This was carried out using human sources of biological protein, i.e. urine and plasma. The decision to use these materials was made for the following reasons, namely, both urine and plasma are readily available, they both contain dilute and concentrated quantities of protein respectively, and finally, and probably the most most important point, the affinity membrane is most likely going to be a useful tool in the pharmaceutical and medical industries, which commonly process these materials on a large scale.

SECTION 4.1 MATERIALS AND METHODS

4.11 - MATERIALS

Tris-HCl (Sigma), sodium hydrogen phosphate (BDH), sodium acetate (BDH), sodium chloride (Aldrich), EDTA (Sigma), acrylamide (Sigma), bis-acrylamide (Sigma), ammonium persulphate (Sigma) TEMED (Sigma), mercaptoethanol (Sigma), glycerol (Aldrich), bromophenol blue (Sigma), glycine (Sigma), Coomassie blue (R250, Sigma), methanol (Aldrich), glacial acetic acid (Aldrich), urokinase (66/46, Institute for Biological Standards), p-Glu-Gly-Arg-pN (Sigma), aprotinin (Sigma), sodium carbonate (Sigma), copper sulphate (Aldrich), sodium potassium tartrate (Sigma), Folin reagent (Sigma), gradient gels (P4/30, Pharmacia), dialysis bags (1" dia).

Filter holder (49mm, Sartorius), peristaltic pump (101 UR, Watson Marlow), spectrophotometer (Uvicord II, 2238, LKB), chart recorder (CR500, JJ Instruments), heating bath (Grant) cooling unit (Grant) gel electrophoresis apparatus (GE-24/LS Pharmacia) power supply (Eps-500/400 Pharmacia), pH monitor (Alpha 500) : Hplc : pressure monitor (803C, Gilson), dynamic mixer (811 Gilson), pumps (303 Gilson), spectrophotometer (Holochrome, Gilson), fraction collector (201, Gilson), chart recorder (N2, Gilson), incubator shaker (New Brunswick Scientific), ultrafiltration apparatus (Millipore), sample freeze drier.

4.12 - METHODS

PLASMA FRACTIONATION - USING THE METAL CHELATE CELLULOSE MEMBRANES

Human plasma (1 ml) in Tris-HCl (50ml, 0.25M, pH8) was pumped through a fixed bed of chelate activated copper membranes (49mm dia, 1g dry wt) held in a Sartorius stainless steel filter holder. The flow was maintained until the concentration of protein emerging in the eluate was approximately equivalent to 95% of the feed solution concentration. The material was then washed with loading buffer and the bound protein desorbed using a stepwise ionic and pH gradient consisting of the following buffer systems: a) sodium hydrogen phosphate (30ml, 0.25M, pH7.5), b) sodium hydrogen phosphate (25ml, 0.25M, pH6.5), c) sodium acetate (2.0mls, 0.25M, pH6). Each of the buffer solutions also contained sodium chloride (0.35M). The eluted protein was dialysed, freeze dried and stored at -30^{o}C until it was required for analysis.

This procedure including the elution protocol was repeated using the zinc activated cellulose membranes.

URINE FRACTIONATION - USING THE METAL CHELATE CELLULOSE MEMBRANES

Freshly voided urine (4 litres) was centrifuged to remove the copious amounts of unidentifiable debris, and concentrated using an ultrafiltration unit which had a molecular weight cut off value of 10,000 K daltons. The resultant solution (100ml pH8) was then processed in the same manner as the plasma sample. After circulating the fluid for six hours the membranes were washed with Tris-HCl (0.25M, pH8). Desorption of the bound protein was once again accomplished using a stepwise pH and ionic gradient incorporating the following buffer systems: a) sodium hydrogen phosphate (0.25M, pH7.5), b) sodium hydrogen phosphate (0.25M, pH6.5), c) sodium acetate (0.25M, pH6.5), d) sodium acetate (0.25M, pH5.5). Buffers a,b,c also contained sodium chloride (0.35M). Buffer d, sodium chloride (0.5M).
The samples were dialysed, freeze dried, and stored at -30^{o}C until they were required for electrophoretic analysis.

DIALYSIS OF THE ELUTED PROTEIN SAMPLES

The protein solutions were poured into dialysis bags and placed into distilled water (5 litres). Equilibration was allowed to take place over a period of 3-4 hours; after which time the water was changed. This process was repeated several times.

SAMPLE FREEZE DRYING

The dialysed solutions were transferred to round bottom flasks (50ml) and frozen in liquid nitrogen, after freeze drying over a period of 18 hours; the solid material was stored at -30^{o}c.

4.13 - GEL ELECTROPHORETIC ANALYSIS OF THE PROTEIN SAMPLES.

PREPARATION OF A 7.5% POLYACRYLAMIDE ELECTROPHORESIS GEL FOR USE IN A DISCONTINUOUS BUFFER SYSTEM.

STACKING GEL PREPARATION

A 2.5ml solution of acrylamide-bisacrylamide (30%/0.8% respectively , in water) was added to a mixture containing Tris-HCl (5ml, 0.125M, pH6.8), 10% SDS (0.2ml) 1.5% ammonium persulphate (1ml), water (11.3ml) TEMED (0.015ml).

RESOLVING GEL PREPARATION

A 7.5ml solution of acrylamide-bisacrylamide (30%/0.8% respectively, in water) was added to a mixture comprising of Tris-HCl (3.75ml, 0.375M, pH8.8), 10% SDS (0.3ml), 1.5% ammonium persulphate (1.5ml), water (16.95ml) TEMED (0.015ml). The resolving gel was poured between two glass plates spaced 2mm apart sealed along the sides with Marprene^{R} tubing. Tris-HCl (2ml, 0.375M, pH8.8) was added dropwise onto the surface of the resolving gel at which time polymerisation began to take place. After polymerisation, the overlay was poured off, and the stacking gel added, into which a spacing comb was inserted. This assembly was left undisturbed until the system had polymerised. The comb was then removed to expose a set of wells which would eventually hold the protein samples.

PROTEIN SAMPLE PREPARATION

Each protein fraction (1mg) in water (0.5ml) was boiled in a solution of Tris-HCl (0.5ml, pH6.8) containing SDS (2%), 2-mercaptoethanol (5%), glycerol (10%) and bromophenol blue (0.002%).

ANALYSIS OF THE PROTEIN SAMPLES

The polyacrylamide gel was placed into a Tris-reservoir buffer solution (5 litres, 0.35M, pH8.3) containing glycine (0.192M). The protein samples were then carefully loaded into each well (50 γl). A current was applied across the gel, [40 mA] which provoked the migration of the protein down through the polymer matrix.

STAINING THE SAMPLE GEL

The gel was removed from the glass plates and placed into a staining solution of Coomassie blue R250 (0.1%) in water : methanol : glacial acetic acid (5:5:2 by volume). The system was left to stain overnight.

DESTAINING THE SAMPLE GEL

The stained gel was transferred to a solution of methanol : glacial acetic acid (7:3 by volume) and left until suitably destained.

The sample preparation, analysis destaining and staining procedures performed on the P4/30 gradient gels were the same as those used on the 7.5% system.

4.14 - UROKINASE ANALYSIS

The hplc analysis of urokinase was performed in the following manner. A Memsep^R unit containing seventy p-ABA activated cellulose membranes was equilibrated with sodium dihydrogen phosphate (0.5M, pH7). Urokinase (3mg, Sp.activity 3200 IU/mg) was dissolved in this solution (0.1ml) and injected onto the top of the cartridge. After washing the system with loading buffer, the bound protein was partially eluted with sodium acetate buffer (0.25M, pH4) containing sodium chloride (0.25M). The eluate was monitored for protein content by absorbance at 280nm. Aliquots were taken (0.025ml) from each fraction (0.25ml) and assayed for urokinase activity.

4.15 - UROKINASE ASSAY PROCEDURE [Chase 1985]

The urokinase activity was determined by monitoring its amidolytic effect on pyro-Glu-Gly-Arg-para-nitroanilide. A sample of each eluate fraction (0.025ml) was added to a solution of Tris-HCl (0.950ml, 0.05M, pH8.8) containing sodium chloride (0.04M) and aprotinin (10 kallikrein units/ml). After incubating this mixture in a water bath at a temperature of 37^{0}C, the chromogenic substrate was added (0.025ml). The rate at which p-nitroaniline was released, was monitored spectrophotometrically at 405nm. The change in optical density was linear with increasing urokinase activity, in the range 5-240 IU in the assay solution. Standard curves were constructed using a urokinase preparation of known proteolytic activity (activity 4,800 IU).

4.16 - PROTEIN ASSAY (LOWRY METHOD)

The protein concentration of each urokinase fraction was measured using the Folin phenol reagent.

Each sample (0.025 ml) was added to sodium hydroxide (0.1 ml), and hydrolysed at 100^{o}C for 10 minutes in a boiling water bath. The hydrolysate was cooled and added to a freshly mixed solution of complex forming reagent (0.8 ml). This system was prepared by mixing the following 3 stock solutions A, B and C in the proportions 100 : 1 : 1, respectively.

Solution A : 2% (W/V) Sodium carbonate in distilled water.
Solution B : 1% (W/V) Copper sulphate - pentahydrate in distilled water.
Solution C : 2% (W/V) Sodium potassium tartrate in distilled water.

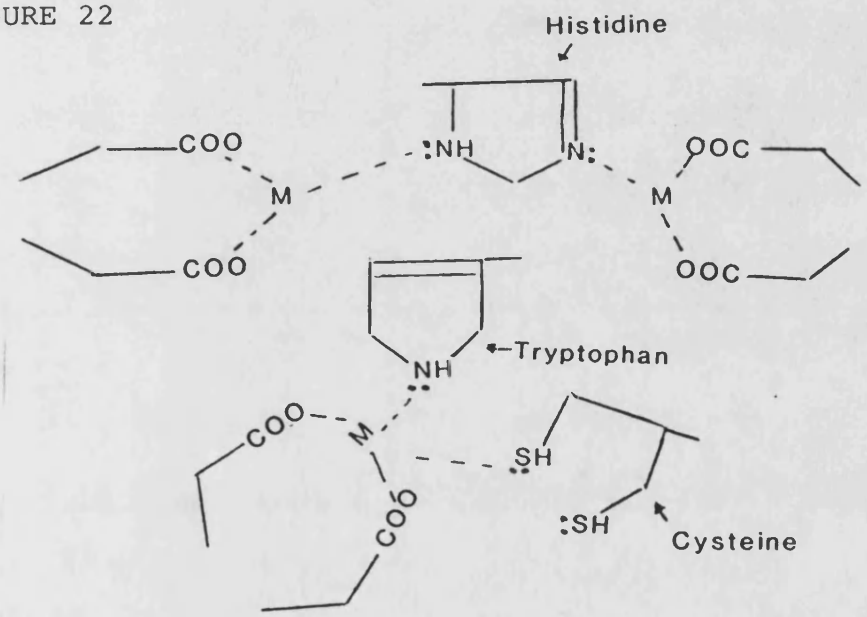
After allowing the resultant complex to stand at room temperature for 10 minutes, Folin reagent (0.1 ml) was added. This was left to react for a further 40 minutes.

The absorbance was then read at 550mm. Standard curves were generated using standardised B.S.A. protein solutions.

SECTION 4.2 INTRODUCTION TO METAL CHELATE CHROMATAOGRAPHY

The original concept of metal chelate affinity chromatography first introduced by Porath [1975] was actually an extension to the earlier work he conducted on molecular sieving and bioselective adsorption [1959, 1968]. It is now generally accepted that the underlying principle of this technique lies with the ability of the electron donor residues cysteine, histidine and tryptophan, which lie on the macromolecular surface of a protein, to form stable coordinate bonds with suitable metal ions. [Fig 22], [Lonnerdal 1982, Sulkowski [1982].

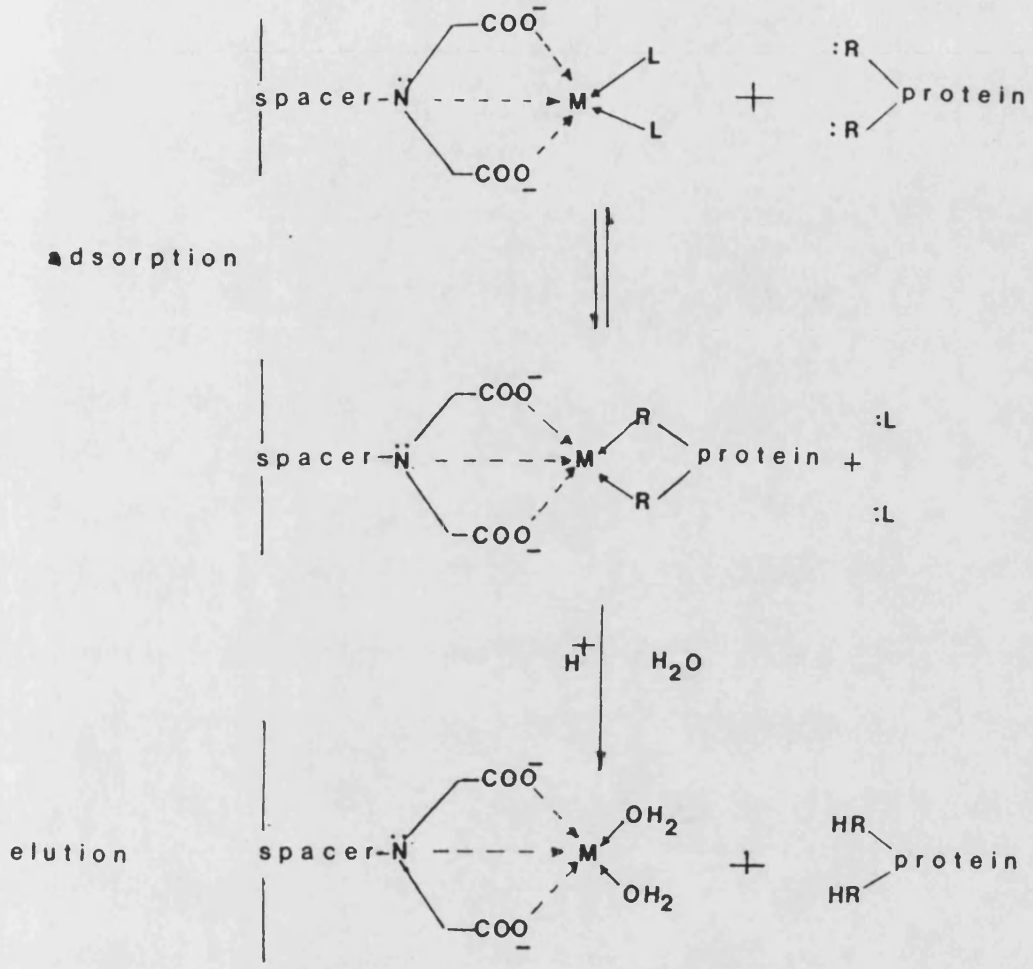
FIGURE 22



Although one could possibly interpret the protein-metal interaction as an ion-exchange effect, Porath [1983] re-enforced the suggestion of a coordinate bond by demonstrating the formation of these complexes at high salt concentrations. This would be an extremely unlikely event if the predominant force of attraction was ionic.

The process of protein immobilisation is normally performed under basic conditions. This is dictated by the anionic nature of the functional amino acids. Consequently destabilisation of the coordinate bond is accomplished using a decreasing stepwise or continuous pH gradient [Scheme 13].

SCHEME 13



If for reasons of gel stability or enzyme activity this protocol is found to be unsuitable, one may alternatively desorb the protein at neutral pH, by adding a competitive ligand to the elution buffer. Imidazole is commonly used for this purpose. Unfortunately this molecule also forms an extremely stable coordinate complex, which is difficult to remove in subsequent washing stages.

If both protonation and competitive elution prove to be unsuccessful, addition of a strong chelating agent such as EDTA will destroy the gel-metal interaction. This will purge all bound substances from the matrix, with consequently very little purification.

SECTION 4.3 PLASMA FRACTIONATION

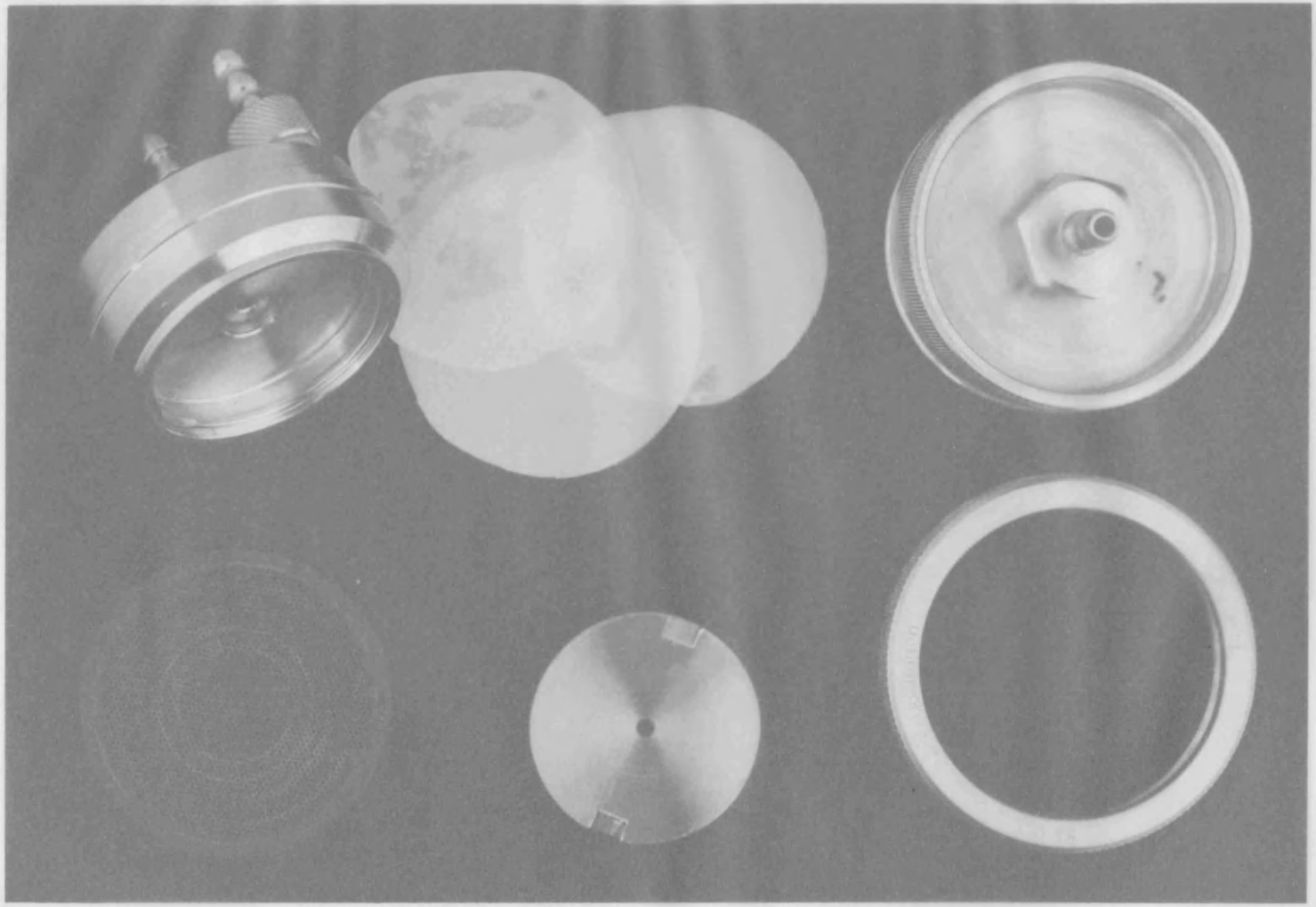
From albumin to zymoplastic factor, the alphabet of plasma proteins has expanded into an evergrowing list. Over the years the theory and practice of plasma protein fractionation has been reviewed with rigour, and has led to the identification of over 200 serum components, (excluding hormones and erythrocyte products). Many of these still do not have a recognised biological function, [Sober 1965, Pennel 1960]. Affinity chromatography offers the analyst a unique opportunity to prepare clinically pure plasma products. This is an extremely relevant point considering hundreds of haemophiliacs have inadvertently injected themselves with the AIDS virus from contaminated samples of Factor VIII.

The zinc and copper chelate membranes discussed in section 2.5 were individually loaded into two membrane cartridges [Fig 23] that were coupled in sequence to a peristaltic pump, u-v spectrophotometer and chart recorder [Fig 24]. Pooled human plasma was passed through this affinity unit until the concentration of protein emerging in the eluate was approximately 95% of that in the feed solution. The material was then washed with loading buffer, and the bound protein desorbed using a stepwise pH gradient [Fig 25, 25a]

Each protein fraction was dialysed, freeze dried and subjected to electrophoretic analysis [Fig 26] from which tentative protein assignments were made [Table ii].

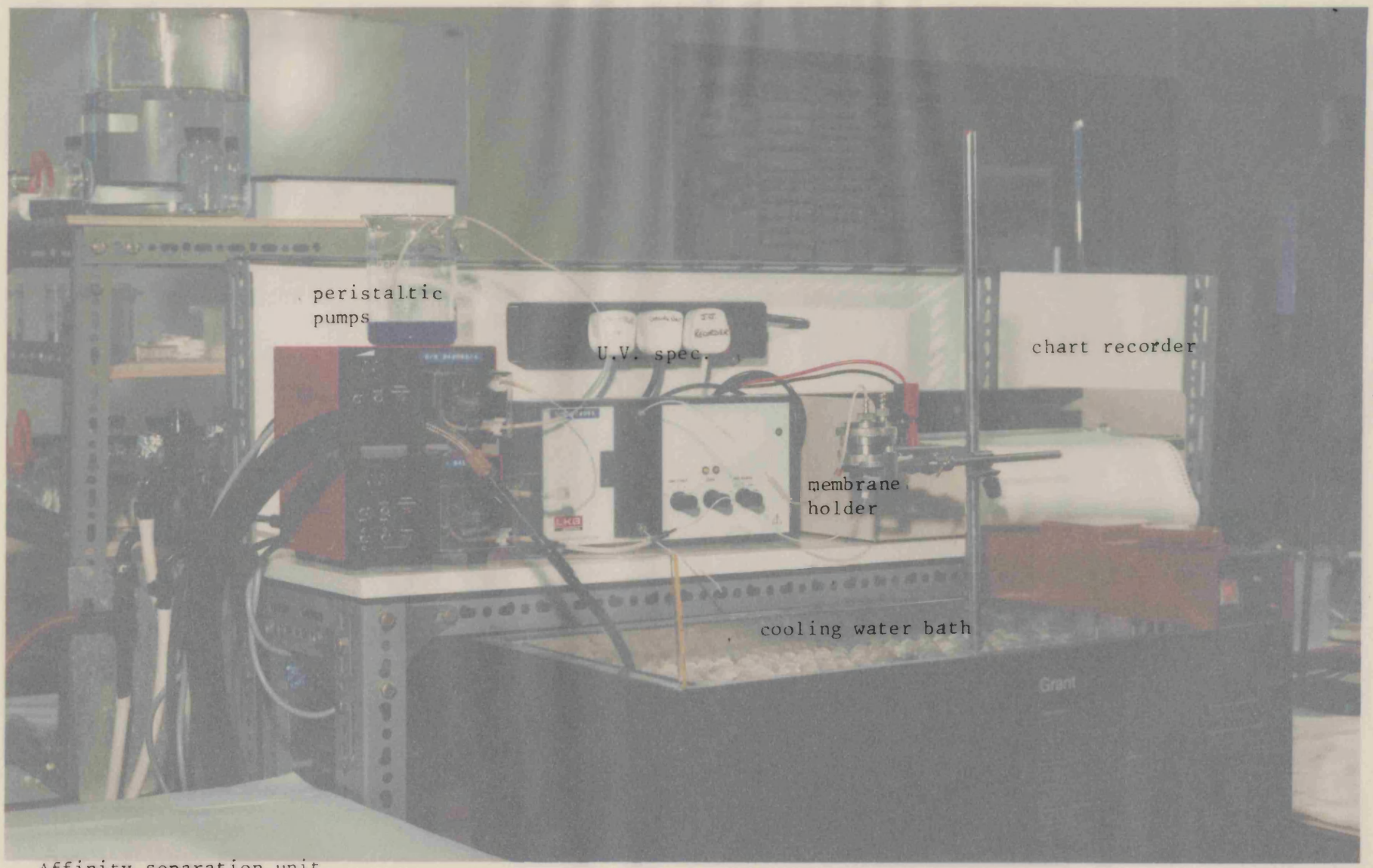
26a

Figure 23



Sartorius membrane filter holder.

Figure 24



Affinity separation unit.

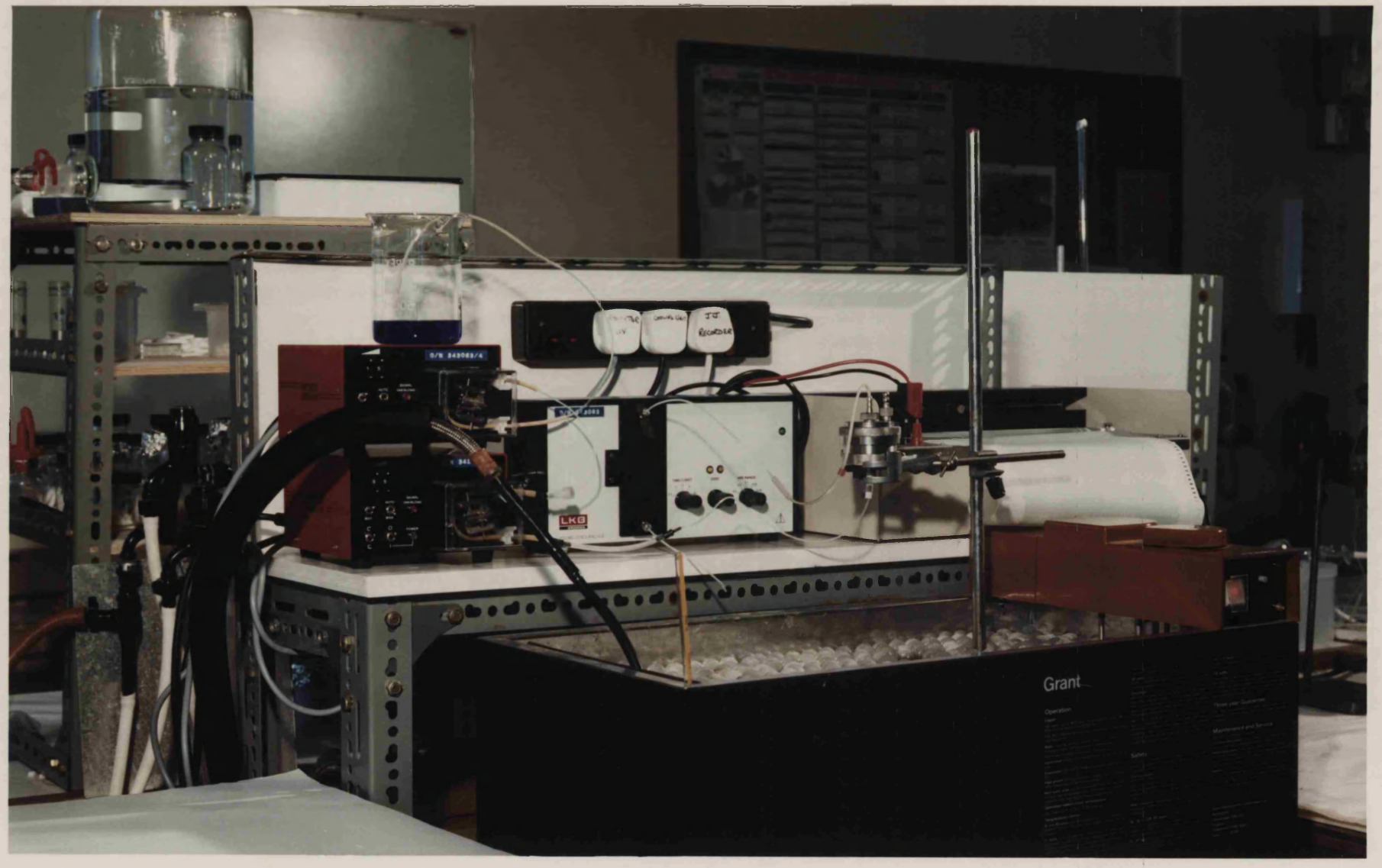
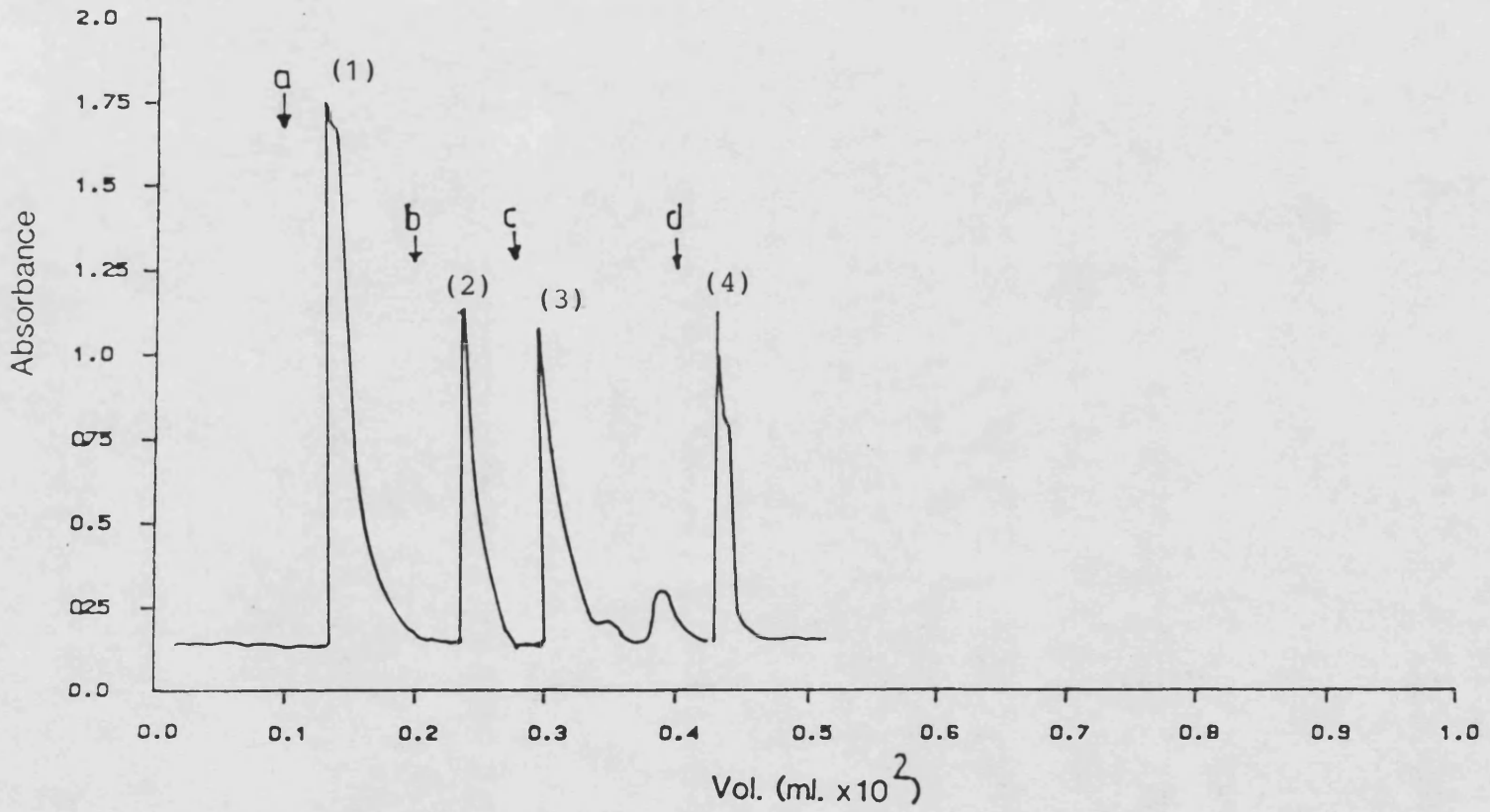
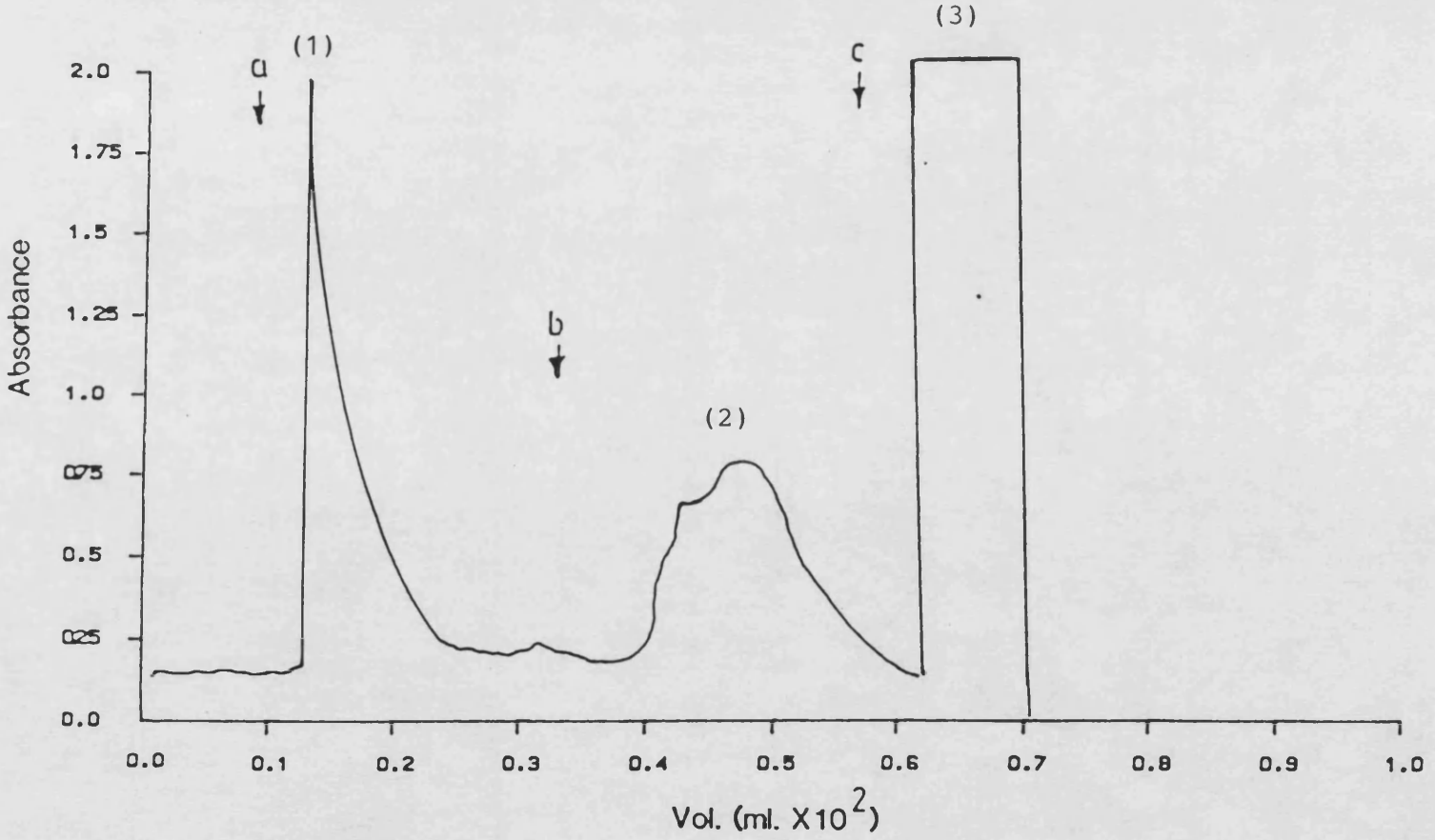

LKB
Grant

Figure 25



Desorption profile of plasma-bound protein from a zinc chelate membrane cartridge (for explanation of buffers a, b, c, d see experimental section 4.12)

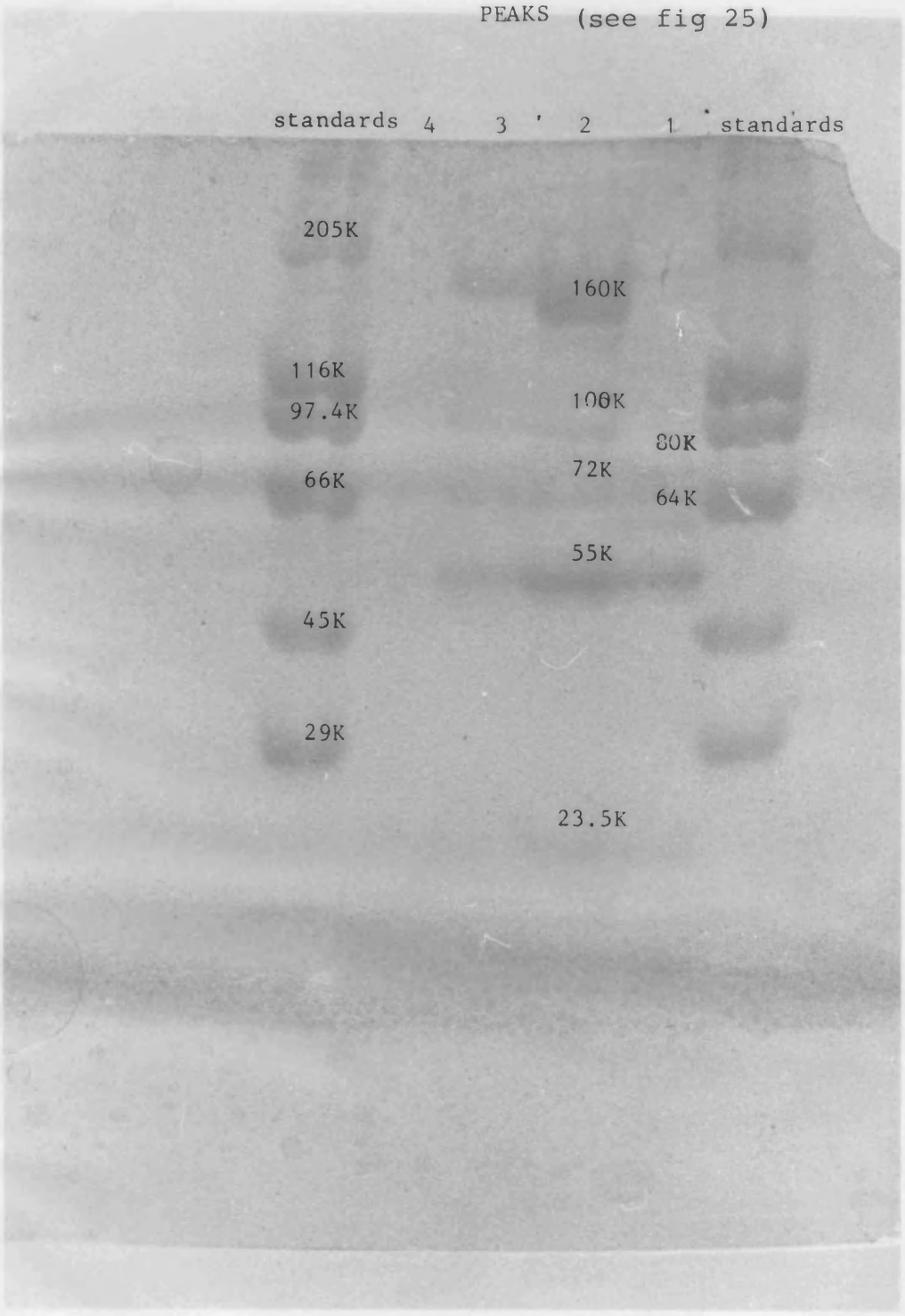
Figure 25a



Desorption profile of plasma-bound protein from a copper chelate membrane cartridge

(for explanation of buffers a, b, c see experimental section 4.12)

Figure 26



Plasma protein eluted from the zinc chelate membranes.
(using a 7.5% slab gel)

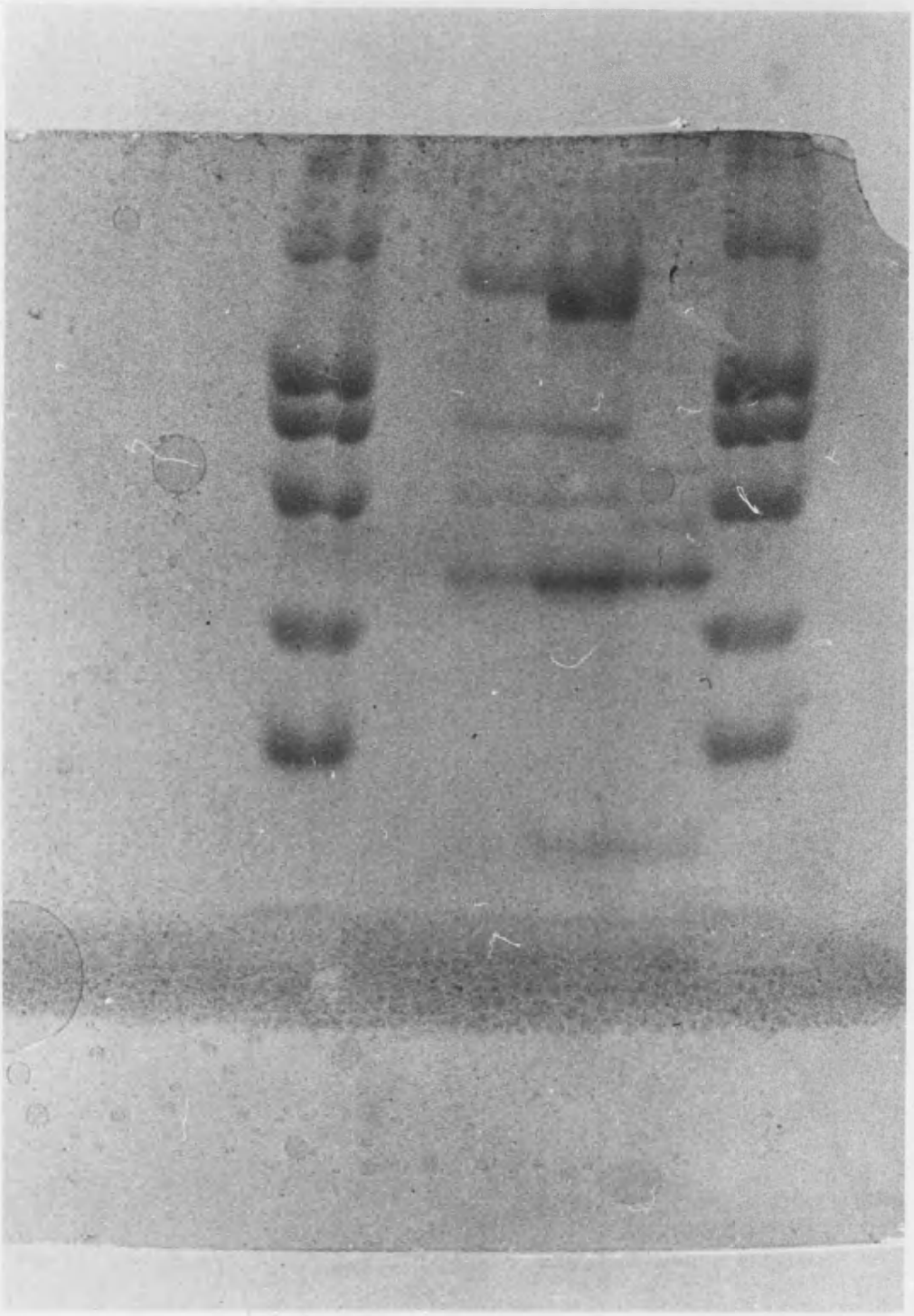


Figure 26a

Plasma protein eluted from the copper chelate membranes.

(using a 7.5% slab gel)

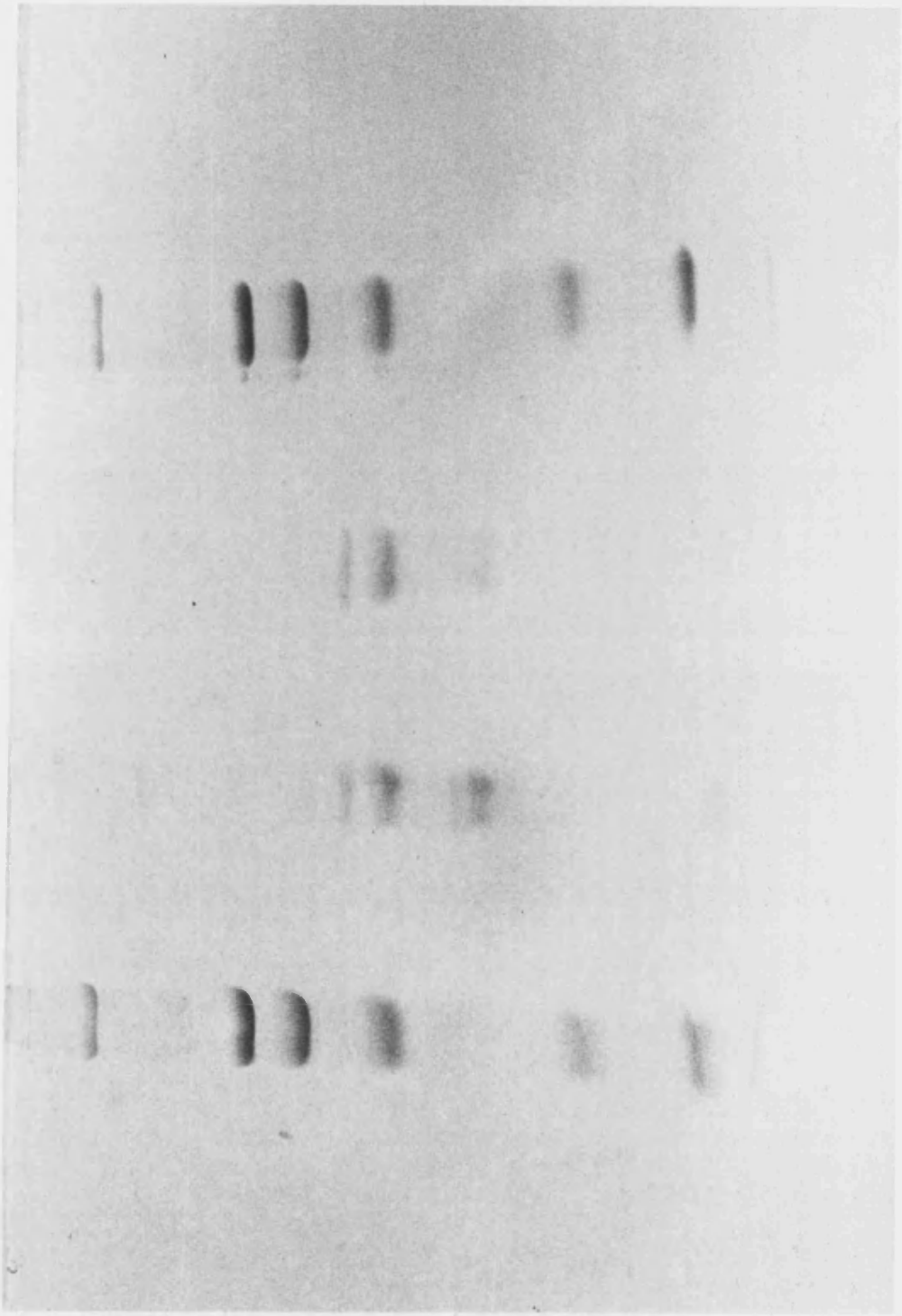


TABLE 11

M.W.	COPPER CHELATE	ZINC CHELATE
160K-140K	IgG	IgG
80K	Transferrin	Transferrin
66K	Albumin	
52K	α - antitrypsin	α - antitrypsin

Although the copper and zinc membranes bound similar M.W. proteins, the latter was distinct in its reduced capacity to bind H.S.A. In his early studies Porath (1975) successfully fractionated human plasma using a zinc and copper chelate constructed from a bead polysaccharide. The protein fractions were identified by gel electrophoresis. In these investigations he also found that H.S.A. preferentially bound to the copper matrix. This has since been confirmed by Hannson [1981]. Later developments have argued, that to successfully immobilise a protein onto a zinc chelate it must contain at least two proximal histidine residues, [Sulkowski 1985]. To demonstrate that this preferentiality was due to the amino acid composition of H.S.A. and not attributable to an inhibitory or competitive force exerted by the other plasma components, the experiment was repeated using a pure preparation of the protein. The pure product also failed to bind to the zinc membrane.

To substantiate the contention that the adsorption process was occurring through a specific interaction with the metal centre, with minimal non-specific influences, the experimental procedures were also performed using membranes devoid of a metal atom as a control. By measuring the areas under the elution profiles it was apparent that less than 10% of the total protein adsorbed was due to non-specific effects.

The enhanced stability of the Cu(II) complexes is reflected in the low pH conditions required to purge the majority of bound protein. This might be attributed to a Jahn-Teller distortion effect which is commonly associated with 6-coordinate copper (II) complexes [Huheey 1972].

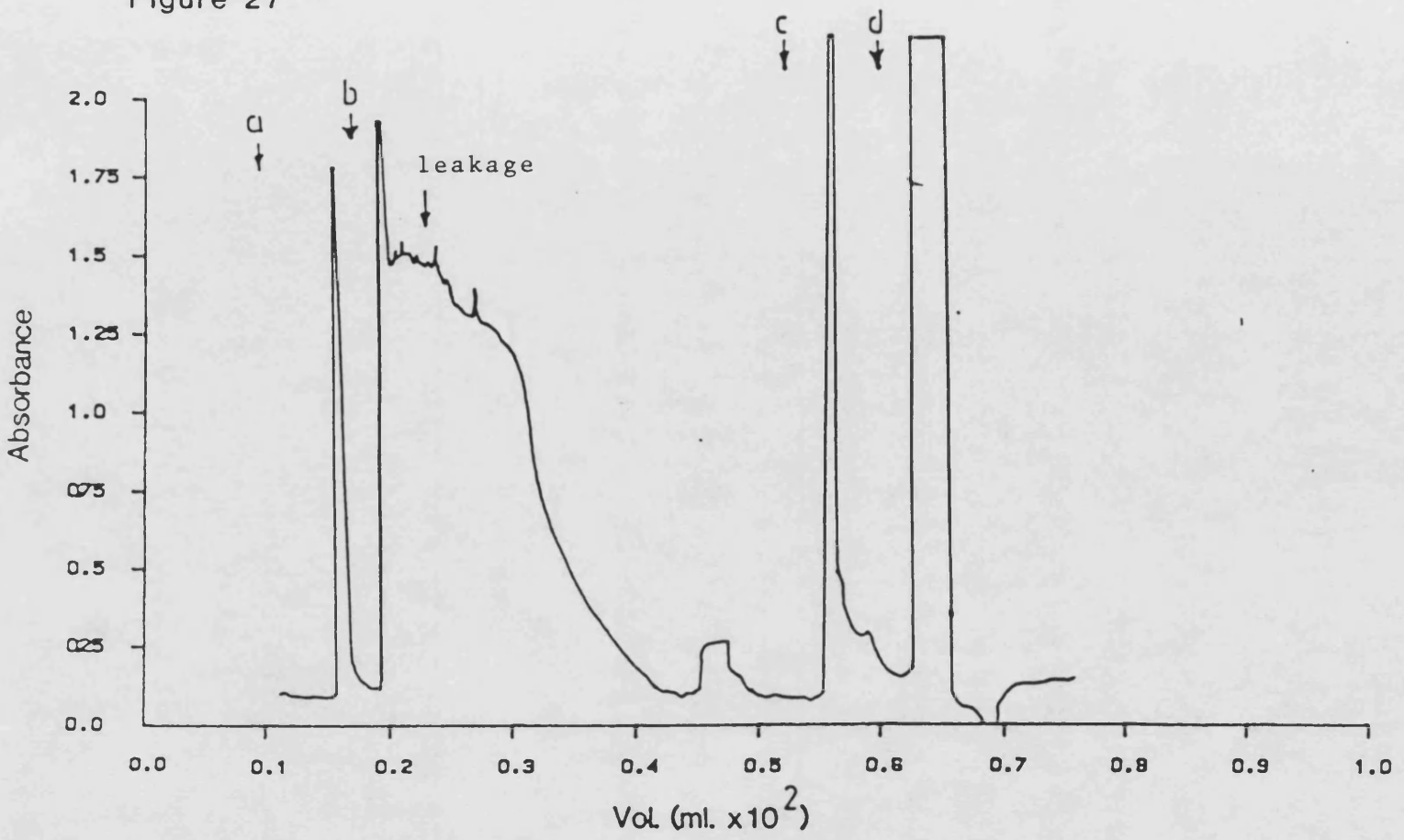
SECTION 4.4 URINE FRACTIONATION

Urine is water containing the water soluble waste products from the blood stream via the kidneys. It consists of 95% water, the remainder being made up of urea, uric acid, creatinine and inorganic salts. The composition does vary widely from day to day. Most importantly urine also contains trace amounts of proteinacious material. The level of various proteins in urine and their relation to renal failure is becoming an area of significant clinical interest. The major urinary protein fraction is plasma albumin which accounts for approximately 40% of the protein material present, globulins make up another 12%. The remaining 48% is composed of lower molecular weight proteins, enzymes, hormones and peptides.

The methodologies of adsorption and elution were similar to those used for plasma fractionation in which a buffered urine solution was recycled through the membrane unit. Desorption was accomplished using a decreasing stepwise pH gradient.

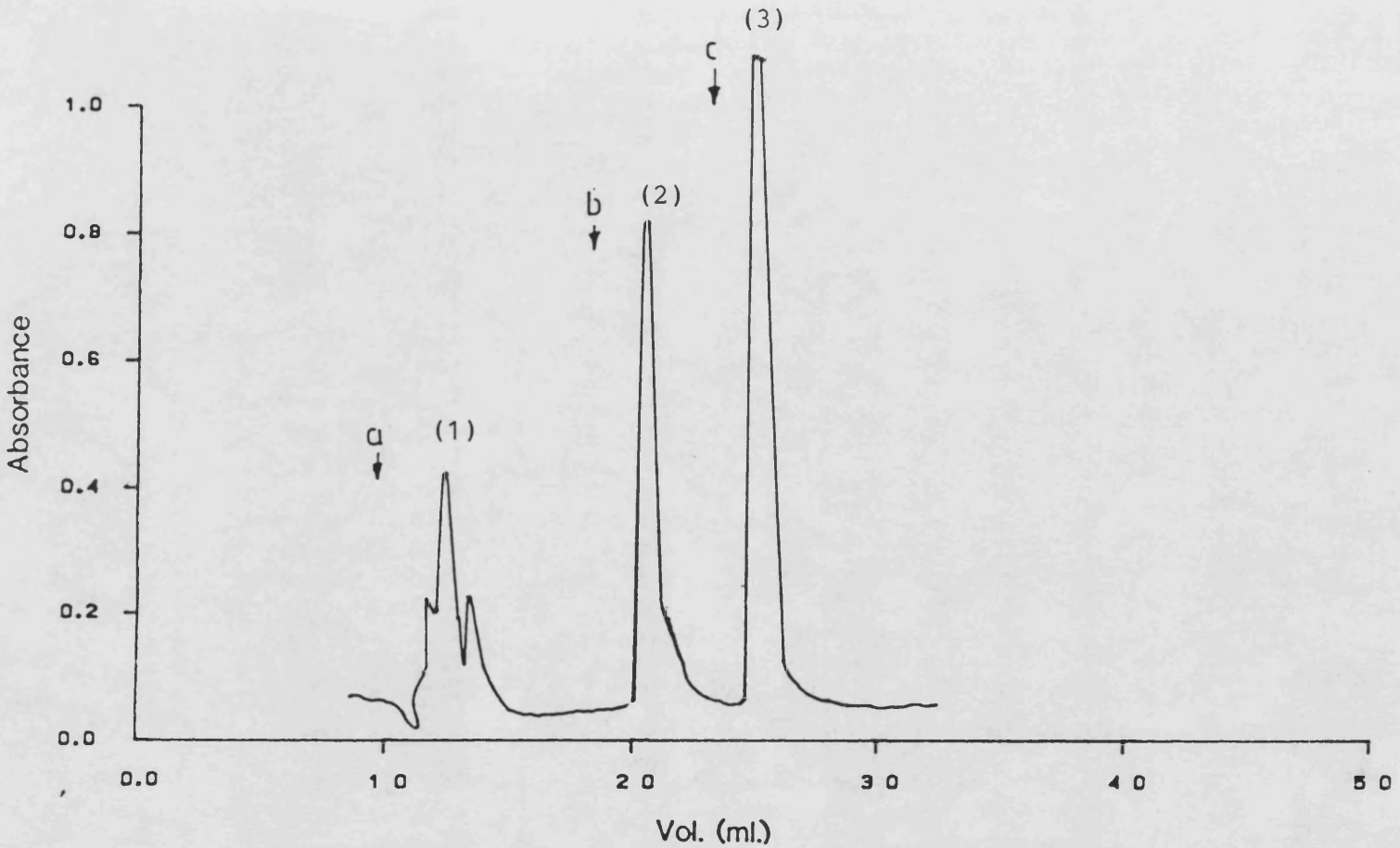
Unlike the plasma study, two problems materialised within this process. The first is highlighted in the copper chelate elution profile, [Fig 27]. As the ionic strength and pH of the buffer was lowered, substantial metal leakage began to occur, which gave a strong blue colouration to the eluate due to the presence of copper. This is indicated by the arrow on Fig 27. There is at present, no suitable explanation for this, although a change in the formulation of the virgin cellulose base material could possibly be a contributing factor. This was prevented in further adsorption studies

Figure 27



Desorption profile of urine-bound protein from a copper chelate membrane cartridge (for explanation of buffers a, b, c, d see experimental section 4.12)

Figure 28



Desorption profile of urine-bound protein from a copper chelate membrane cartridge pretreated with buffer c

(for explanation of buffers a, b, c see experimental section 4.12)

by pre-equilibrating the membranes in a low pH buffer solution to remove the superfluous, loosely bound metal ions. This proved to be extremely effective in subsequent elution procedures.

The second obstacle lay with the propensity of the smaller polypeptides (m.w.<5000) to bind preferentially to the matrix, which, as a result, made the majority of metal sites inaccessible to the larger macromolecules.

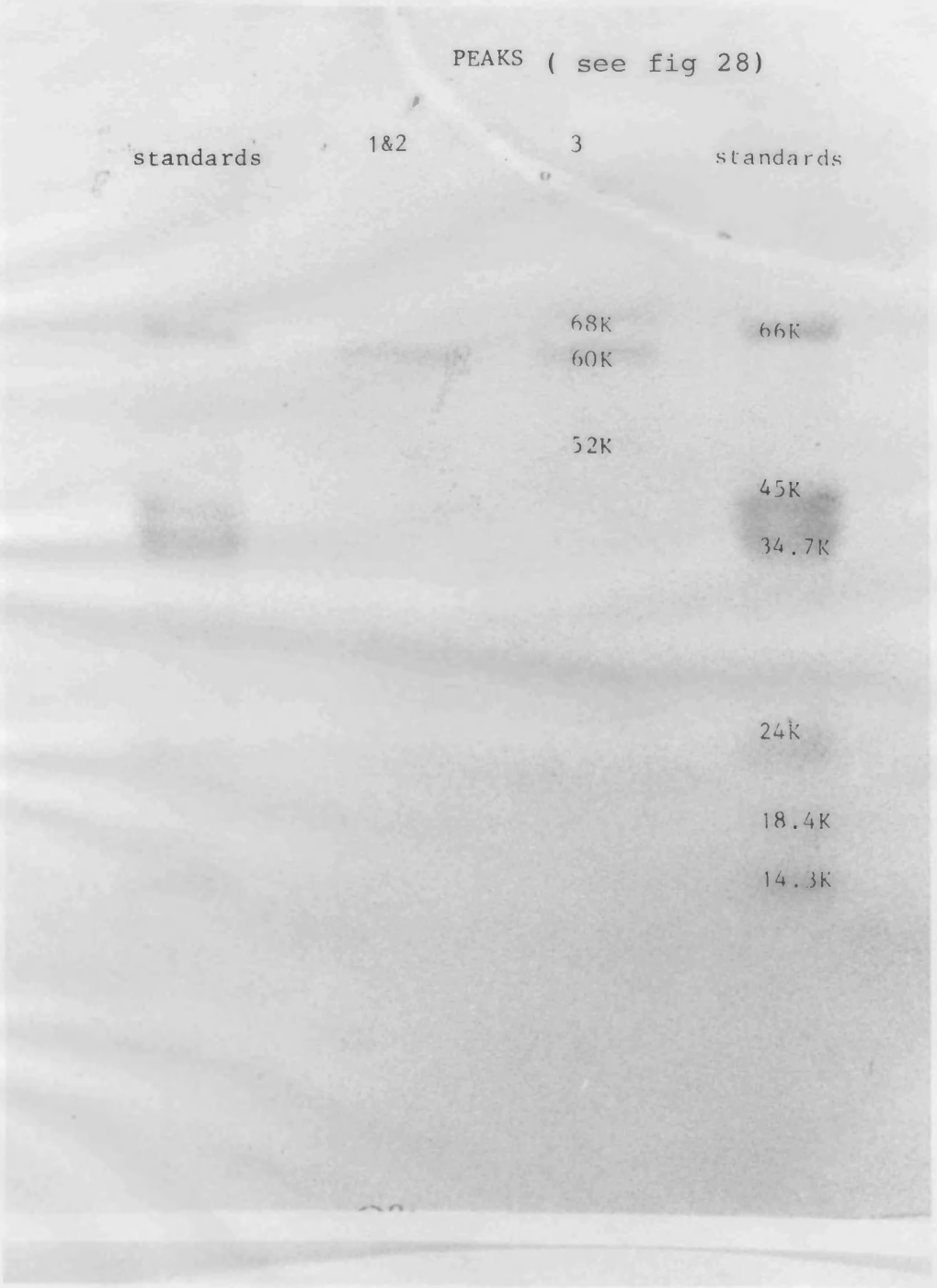
Circulating the urine through an ultrafiltration membrane unit with a molecular weight cut off value of 10,000 seemed to remedy this situation. In fact both pre-treatment conditions made a considerable difference to the pattern of the final elution profile with a subsequent improvement in peak resolution [Fig 28].

The presence of several protein components were indicated by gel electrophoresis, although they were not identified, due to the lack of definitive information at this time [Fig 29].

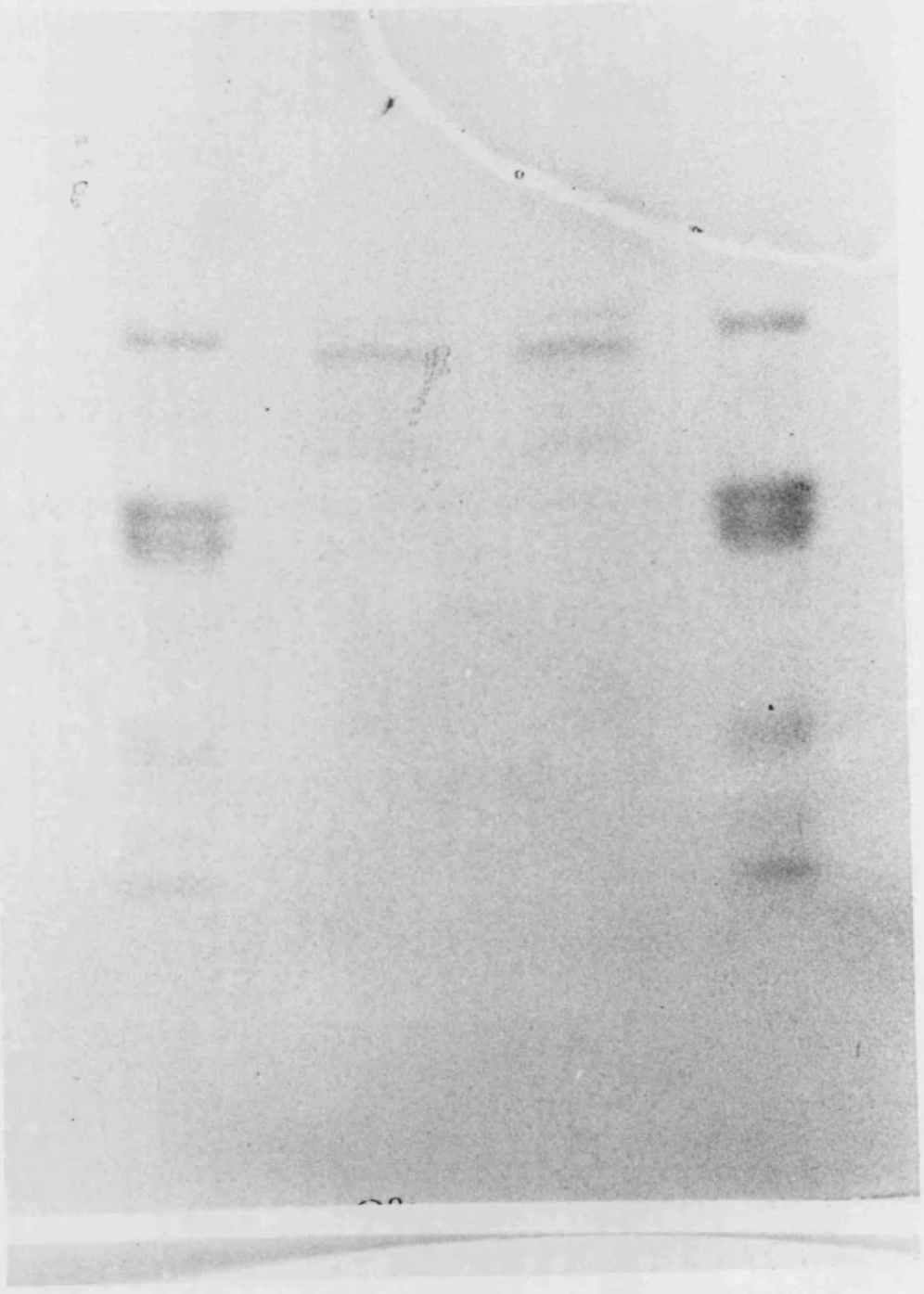
SECTION 4.5: UROKINASE ADSORPTION TO p-ABA ACTIVATED CELLULOSE MEMBRANES

Among the many exotic and expensive materials that have been isolated from urine, perhaps one of the most characterised has been urokinase. This enzyme is a potent fibrinolytic activator which is responsible for the conversion of plasminogen to plasmin [Robbins 1973]. Due to its thrombolytic activity urokinase is used therapeutically to promote the dissolution of thrombi *in vivo* [Matsuo 1982]. Because it appears in such dilute quantities in urine, many time consuming and elaborate isolation procedures have been adopted, including monoclonal antibody techniques (Einarsson 1985, Nakamura 1984]. Many synthetic adsorbents have also been used to prepare homogenous urokinase samples including fibrin celite [Husain 1983],

Figure 29



Urine protein eluted from the copper chelate membranes.
(using a p4/30 Pharmacia gradient gel)



aminocaproyl agmentine [Soberano 1976] and arginine-hexylester [Robbins 1983]. Investigations have also indicated that p-ABA is a competent urokinase inhibitor [Chase 1985, Strickland 1983].

The urokinase analysis was performed using a membrane capsule designed by Domnick Hunter Filters Ltd. It accommodated approximately seventy p-ABA discs [25mm dia prepared according to the procedures outlined in section 2.1]. The unit was successfully integrated into a Gilson hplc system [Figs 30, 31].

Three milligrams of protein, total specific activity 3200 IU/mg was loaded onto the membrane cartridge. After washing the system, approximately 75% of this material passed through unadsorbed. These fractions contained no enzymatic activity. Equilibration with sodium acetate buffer eluted 5,800 IU approximately 60% of the total protease activity applied, the remainder could only be removed by adding a 1% solution of SDS to the system. The final product had a total specific activity of 11,600 IU/mg, which corresponds to a 3.5 fold increase in purity.

The membrane unit undoubtedly offered several potential advantages over the more traditional column systems. In addition to being relatively inexpensive to produce, the cartridge should reduce the analysis time (this procedure took approximately 7 mins). The cartridge also provides one with a means to concentrate dilute solutions. The analyte in a large sample could be applied to this system and eluted as a concentrated peak. Finally the analysis could be performed at low pressures, typically of the order of 2-3 bar at a flow rate of 2 ml/min.

Figure 30

Memsep[R] membrane cartridge.

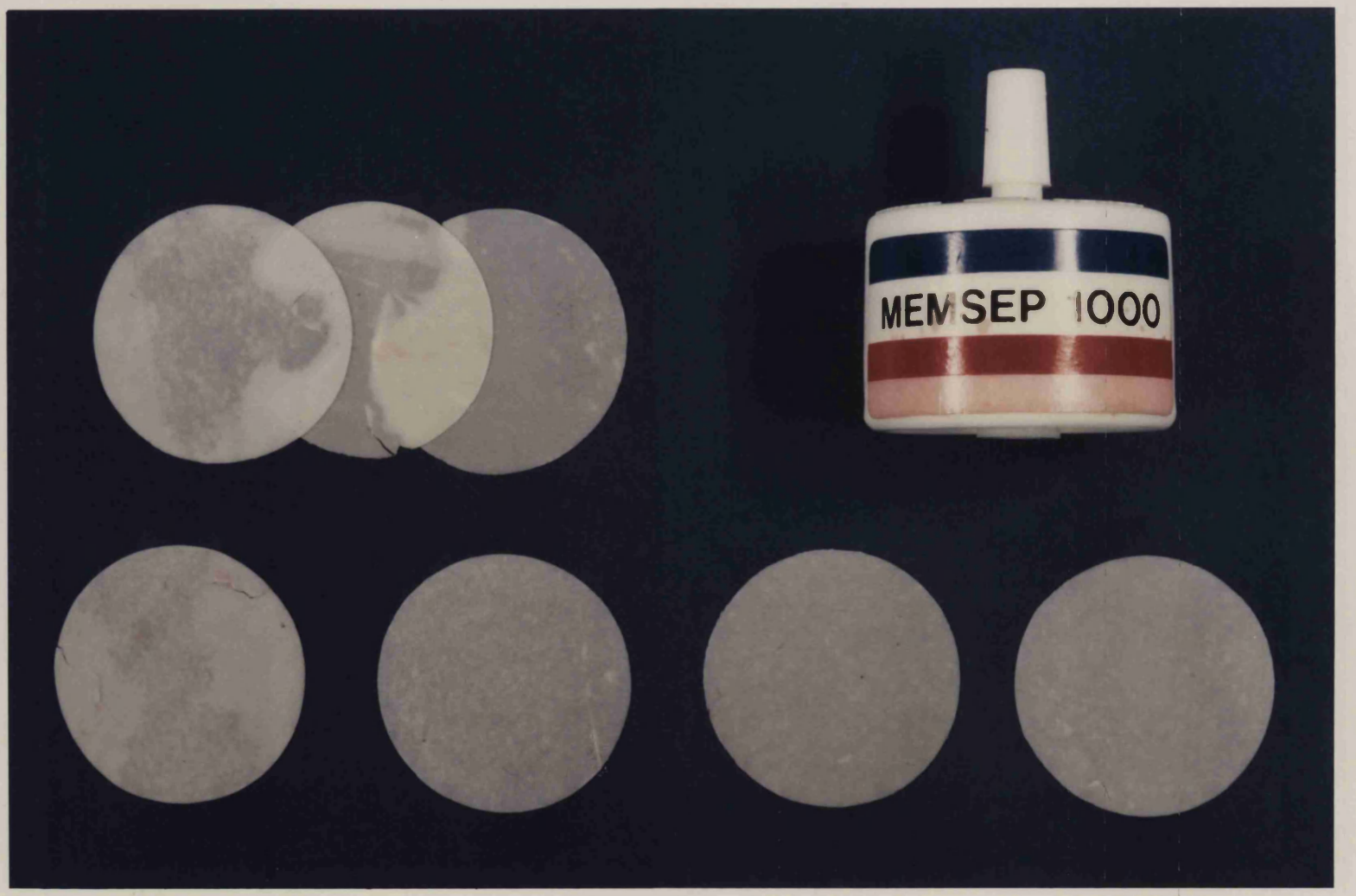

MEMSEP 1000

Figure 31

Sample loading through a Memsep^{R} cartridge.


MEMSEP 10
1
2
3
4
5
6

CHAPTER V: CONCLUSIONS AND FUTURE RECOMMENDATIONS.

The purpose of this study was to develop a novel affinity matrix constructed from an arrangement of cellulose membranes. Two classes of affinant were prepared, a protein selective derivative containing the tryptic inhibitor p-ABA, in conjunction with a metal chelate analogue, a semi-specific protein adsorbent. The materials used to activate the cellulose membranes were relatively inexpensive and the methods of activation were simple to perform. In addition, the concentration of activation sites on the surface of the membrane was accurately controlled by adjusting the initial reagent concentration. The products of coupling are exceptionally stable and can be stored with a suitable bacteriostat for periods of several months without any apparent loss in activity. These advantages have facilitated the convenient scale up of the derivatisation processes which is now being undertaken by Domnick Hunter Filters Limited.

The aspect of quantitative affinity chromatography was illustrated using a simple model to describe the kinetics of the batch adsorption of trypsin onto the p-ABA membranes. Predictions were made regarding the rates of protein adsorption and desorption onto the adsorbent. The variable nature of the K_1 values obtained suggests that the method used to monitor the adsorption-desorption process was inefficient. The time required to sample and record the protein concentrations undoubtedly introduced significant errors into the initial rate of adsorption measurements. This problem could be minimised if in future experiments, the protein solution was pumped through the membranes directly into a spectrophotometer flow cell. This method would also minimise the problems associated with mass transfer. The values of K_{de} and q_m for the batch reactions were however in excellent agreement with the predicted results i.e. the final experimental equilibrium concentrations were similar to those predicted by the computer analysis. The sensitivity of the binding process to changes in the initial reaction conditions was also ascertained. Dramatic increases in the value of K_{de} were induced when the temperature of the reaction was increased or the pH decreased. This translated into a marked reduction in the concentration of bound protein on the affinity membrane.

The metal chelate membranes were used to isolate a range of protein components found in both plasma and urine. A tentative identification of the adsorption products was made using gel electrophoresis. HSA, transferrin and a_1-antitrypsin were believed to be among the plasma components isolated. The material isolated from the urine sample was assigned molecular weight values , but it requires further analysis for a positive identification of the protein constituents to be made. The problems of channeling, compression and blockage usually associated with bead columns were not in evidence during the course of these investigations. The problems of metal leakage encountered with the metal chelate derivatives was minimised using a low pH wash pretreatment procedure. It is felt in this instance further work should be carried out into the nature of the metal ligand interaction, and the influence if any, the base cellulose material exerts on its stability. In a second series of experiments the p-ABA membranes were enclosed within a capsule which was successfully integrated into a hplc system. This unit was used to purify a sample of urokinase. Although one could not elute all the protein activity from the membrane unit a purification factor of 3.5 was attainable. This result was extremely encouraging in light of the commercially expensive nature of the product.

In conclusion the affinity membrane may well prove to be a useful alternative to the granular materials currently employed. Probably one of the major advantages of these derivatives over the latter materials is their inexpensive nature. Because of their low cost one has the choice of using the membranes repeatedly or disposing of them after each purification run. They are convenient to handle, and have good mechanical and chemical stability and the problems associated with channeling and compression have effectively been removed.

In the field of membrane affinity chromatography, there are still many avenues of investigation waiting to be explored.

The number of activation procedures is growing at an ever expanding rate. In addition to the p-ABA and metal chelate derivatives, a series of ion exchange, dye and protein-A membranes have since been prepared by Domnick Hunter Filters Limited. This is by no means a complete list, for

example, the ares of hydrophobic and covalent membrane chromatography have yet to be exploited. The discussion on immobilised metal chelate affinity chromatography highlighted a technique which differentiated between macromolecules on the basis of their histidine content. This property could well serve as a useful probe in elucidating the topography of histidine residues on the surface of a protein.

The variety of the chelating ligand is limited only by the imagination of the experimenter. One useful modification would be to introduce onto the surface of the matrix a " hybrid–multidentate" ligand. These molecules are now well known for their ablity to coordinate both "hard" and "soft" metal ions. This would give one the unique opportunity to study the protein binding process using metals not only from the latter part of the transition metal series, such as copper and zinc, but also from groups IVa and Va.

One interesting proposition in terms of large scale protein isolation is offered by the affinity membrane. If one was to use them in a crossflow arrangement rather than a dead end mode they could be used in the same manner as an ultrafiltration membrane, having the added advantage of greater selectivity. The bulk protein solution would be passed across the surface of a membrane containing the covalently coupled affinity ligand specific for the protein of interest. The solution would be recycled through the system until the binding sites were saturated, whereby the bound protein would be eluted in a second step.

Appendix I

ADSORPTION OF COMMERCIALLY PREPARED TRYPSIN USING A MEMBRANE SUPPORT MATERIAL

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SUMMARY

The ability of a recently developed affinity membrane to adsorb commercially prepared trypsin was investigated. Several buffered solutions of trypsin which varied in their initial concentrations from 62.5 mg/ℓ to 1,000 mg/ℓ, were passed through a stacked bed of seven membranes; dry wt 350 mgs. The adsorbed protein was eluted using acetic acid; 2.2 mgs to 5.3 mgs of trypsin was desorbed. The adsorption capacity tended to a maximum of 16 mg /g dry wt when the initial feed concentration of trypsin was 1 g/ℓ. There was no loss in enzyme activity after desorption; 11,500 IU ± 500 IU.

INTRODUCTION

Affinity chromatography is a form of adsorption chromatography in which the species to be purified binds specifically, strongly and reversibly to an immobilised ligand on an insoluble support. [Dean et al., 1985].

When designing an affinity support one has to consider many factors; most importantly, the type of support material, the ligand to be bound and the method of activation. Each type of matrix displays a variety of physical and chemical properties eg rigidity, porosity stability, and solubility which ultimately determines its suitability as an affinity medium. The desired affinity ligand must be capable of binding specifically and reversibly with the product to be separated, and couple to the matrix in a manner that does not appreciably reduce its activity. It must also possess a chemically reactive group to allow immobilisation onto the matrix, and remain stable throughout the immobilisation procedure. Once the matrix and ligand have been chosen, one finally has to decide upon the method of activation. There are many activation procedures available to bind a ligand to the solid support, eg CNBr, gluteraldelyde, epoxide, etc. Generally one can couple directly onto the support, or to a spacer group which in effect immobilises the ligand away from the matrix.

This work characterises the ability of a p-aminobenzamidine membrane to adsorb commercially prepared trypsin under conditions of a moderately high flow rate and low temperature.

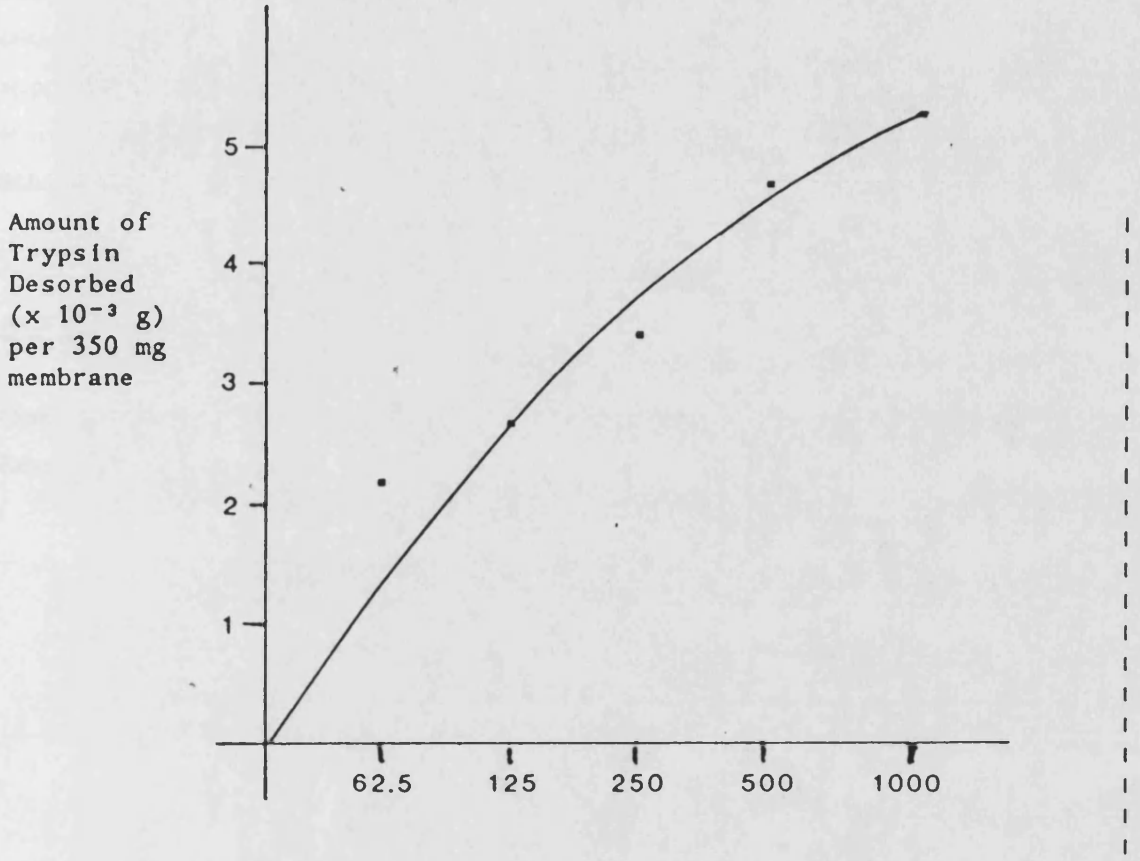
EXPERIMENTAL

The chromatography unit consisted of a Watson Marlow 101UR peristaltic pump, a Sartorius SM 16278 membrane holder, a J J instruments CD 500 chart recorder an LKB 2238 Uvicord SII spectrophotometer and a grant cooling bath. The membranes were supplied by Domnick Hunter Filters Ltd. Trypsin and BAEE were obtained from Sigma chemical C^0. The adsorption and elution buffers were respectively, Tris-HCl. (50 mM, pH 8, + 0.5 M NaCl) and Acetic acid, (100 mM + 10 mM $CaCl_2$). 100 mls of buffered trypsin was passed through a stack of seven 49 mm membrane discs, equilibrated at 4 C with adsorption buffer, at a flux rate of 8.53 x 10^{-2} mm/min.

After one cycle the membranes were washed with adsorption buffer, and the bound protein eluted with acetic acid. The concentration of trypsin emerging in the effluent was measured as a function of its absorbance at 280 nm. The enzyme activity was determined using the substrate BAEE. [Schwert and Takenaka 1955].

RESULTS AND DISCUSSION

P-aminobenzamidine is an affinity ligand commonly used to isolate and purify a range of serine proteases, more commonly trypsin (K_d = 8.25 x 10^{-6}). [Mares-Guia, 1965; Bethell et al., 1981]. Following the procedure of Bethell et al, (1979) it was decided to incorporate this inhibitor onto a membrane to ascertain whether a support material in this form would have an improved performance over the beaded or granular materials currently employed. The adsorption of trypsin with respect to the initial feed concentration is illustrated in Fig.1. The adsorption increases to a maximum with a feed concentration of 1 g/ℓ.



At a flux rate of 8.53 x 10^{-2} mm/sec a steady state equilibrium between enzyme and substrate was attained at the end of one cycle: the final adsorbance of trypsin emerging in the effluent was approximately 90% of the initial absorbance of the feed solutions. At this flux rate the membranes exhibited very little resistance to flow; there was a pressure differential of 1.1 bar across the membrane unit. They also had the added advantage over the granular material of not being succeptible to channeling or compression. Only short equilibration times with buffer were required (<10 min) and the affinity matrix was successfully used after several months of storage.

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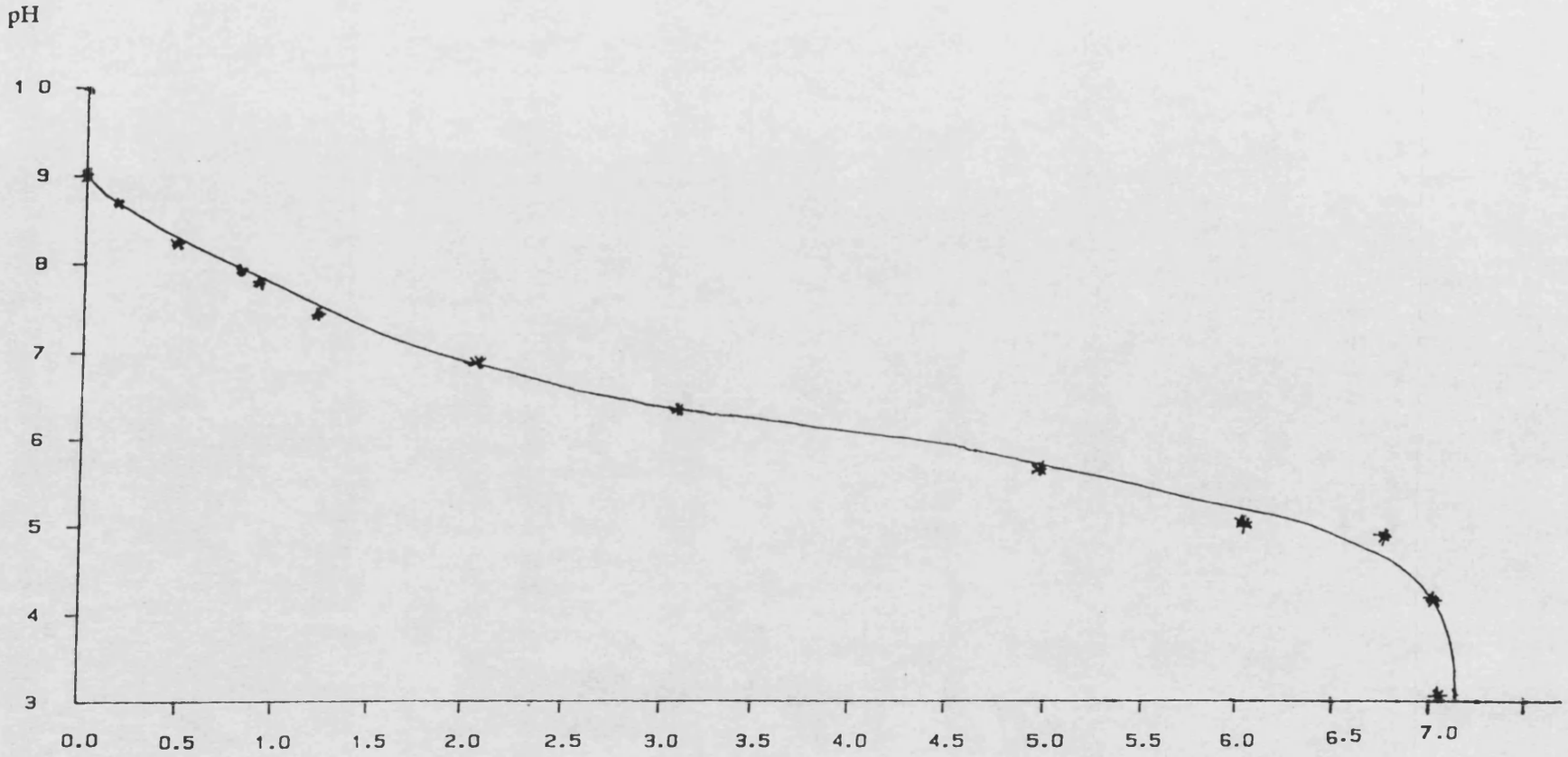
Appendix II

Titration of CDI activated membranes under nitrogen using 0.1M HCl on 3mMol activated cellulose membrane.(p 18)

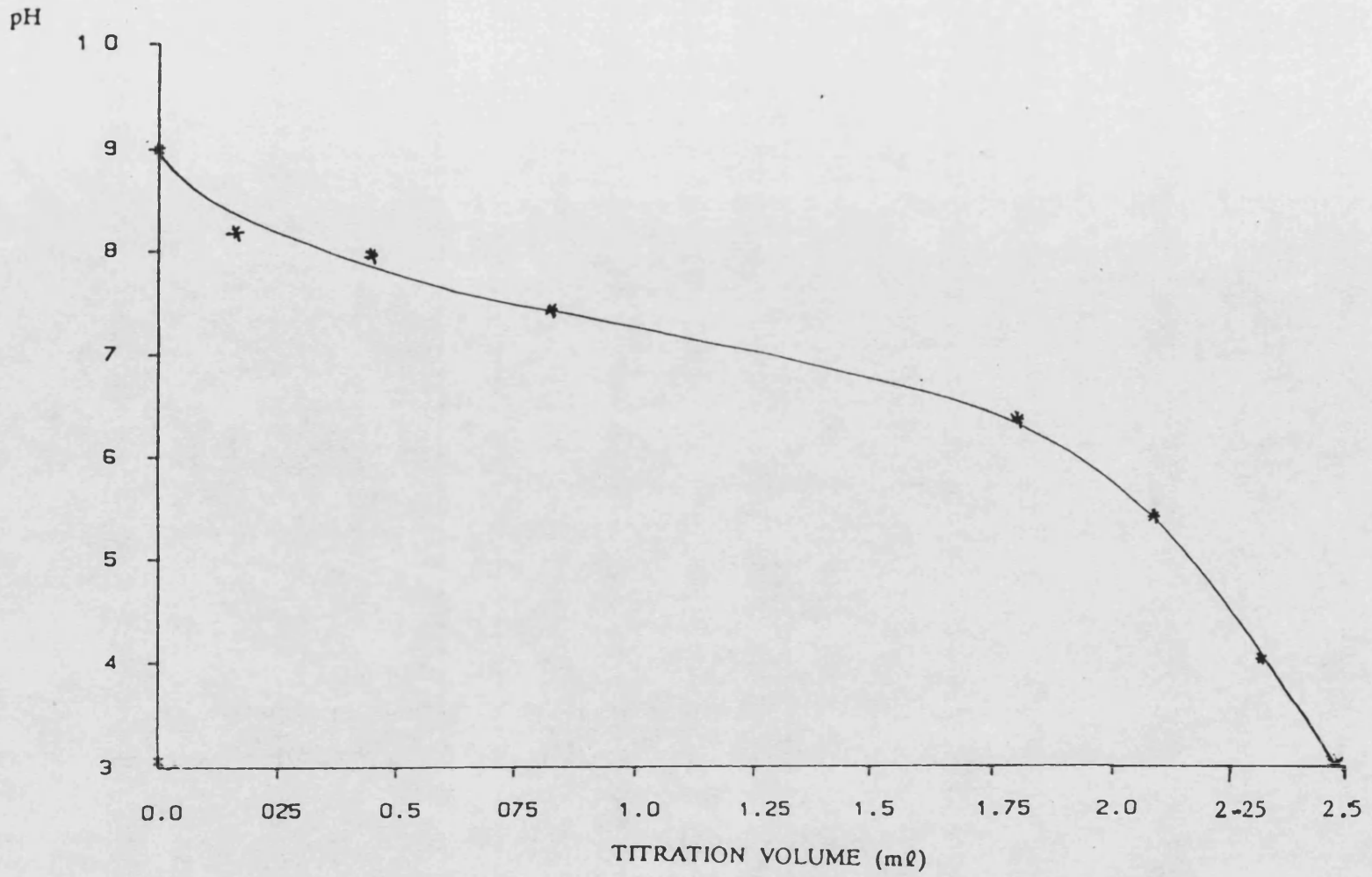
VOL (HCl ml)	pH
0	9.0
0.20	8.7
0.50	8.3
0.70	8.0
0.90	7.7
1.25	7.5
1.65	7.3
2.15	7.0
2.75	6.8
3.15	6.5
4.10	6.3
4.95	6.0
5.75	5.8
6.20	5.5
6.80	4.9
6.95	4.5
7.00	4.2
7.05	4.0



Titration curve of CDI activated cellulose membranes (under N_2)

Titration of CDI activated membranes after removing the nitrogen using 0.1M HCl on 3mMol activated cellulose membrane (p 18)

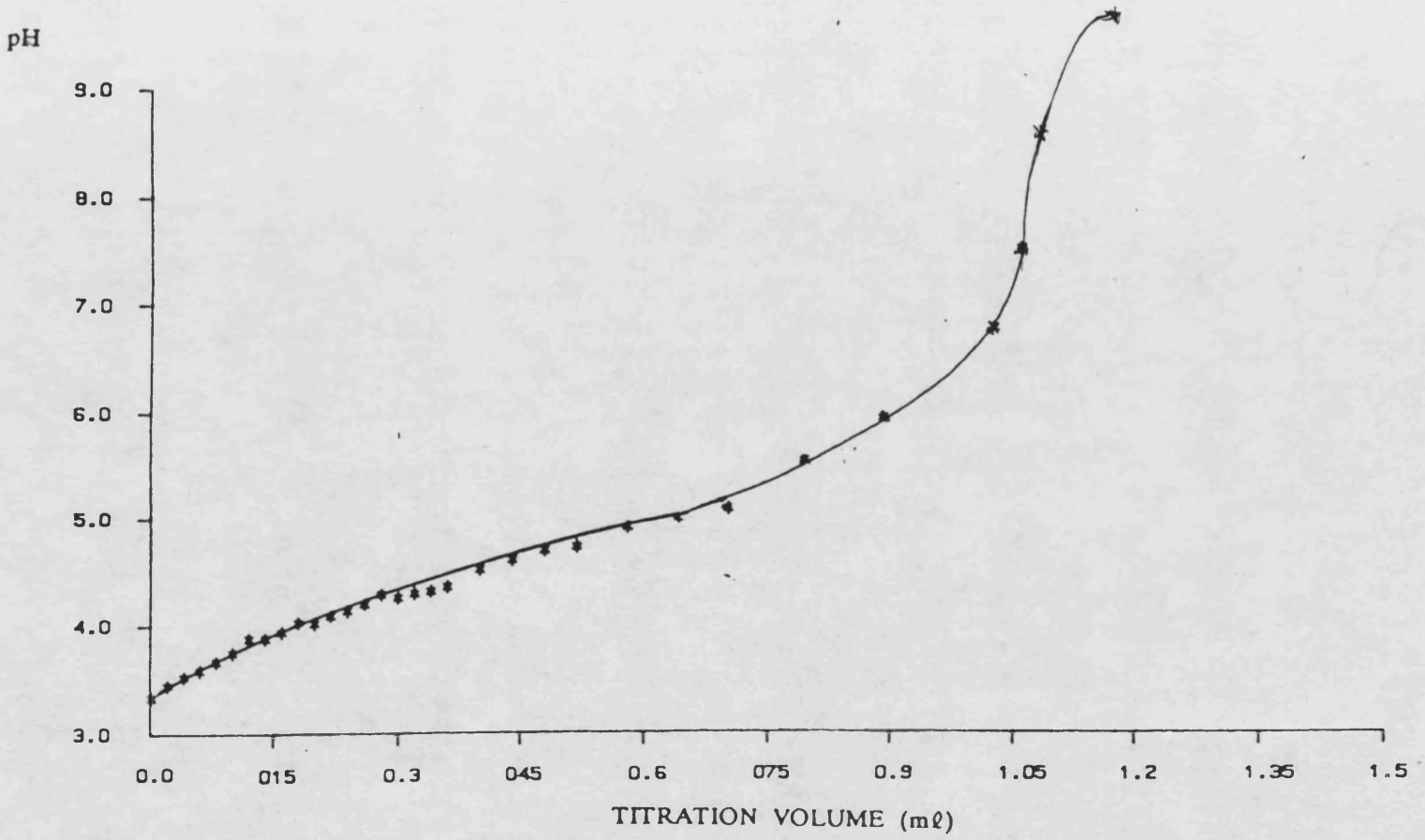
VOL (ml HCl)	pH
0.00	9.0
0.22	8.2
0.44	8.0
0.58	7.7
0.83	7.6
1.60	7.0
1.80	6.5
2.10	6.0
2.20	5.5
2.30	4.3
2.35	4.0
2.50	3.0



Titration curve of CDI activated cellulose membranes
(after blowing off N_2)

Titration of 6-aminohexanoic acid membranes using 0.1M HCl on 3.4 mMol activated cellulose membrane (p 22)

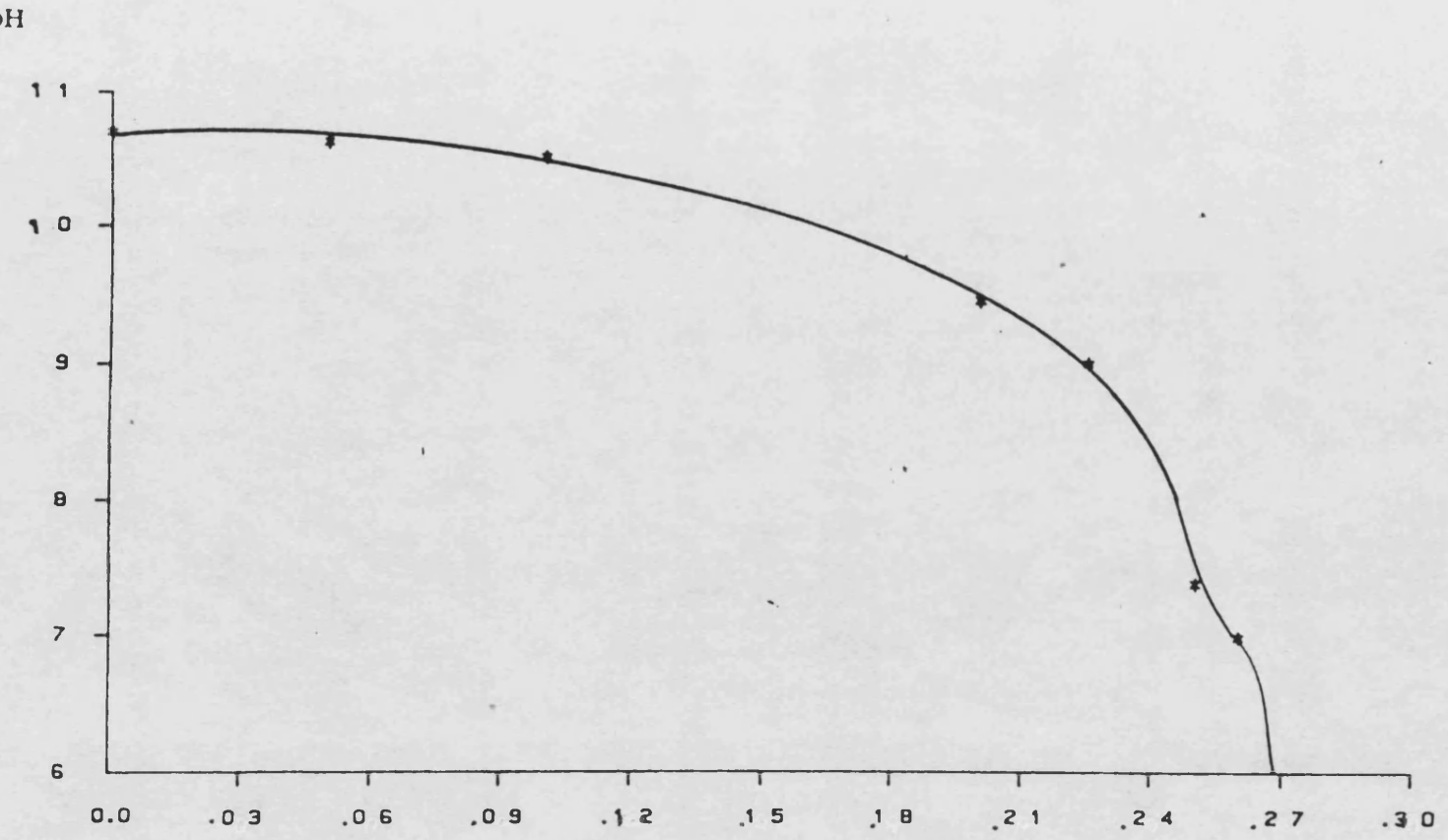
VOL (μl HCl)	pH
0	3.3
20	3.4
40	3.5
60	3.6
80	3.7
100	3.8
120	3.9
140	3.9
160	3.9
180	4.0
200	4.0
220	4.1
240	4.1
260	4.2
280	4.3
300	4.3
360	4.4
400	4.5
440	4.6
480	4.7
580	4.9
640	5.0
700	5.1
800	5.5
900	5.9
1000	6.6
1060	7.6



Titration curve of 6–aminohexanoic acid activated cellulose membranes

Titration of epoxide activated membranes with 0.1M HCl on 20% activated cellulose membranes. (p 28)

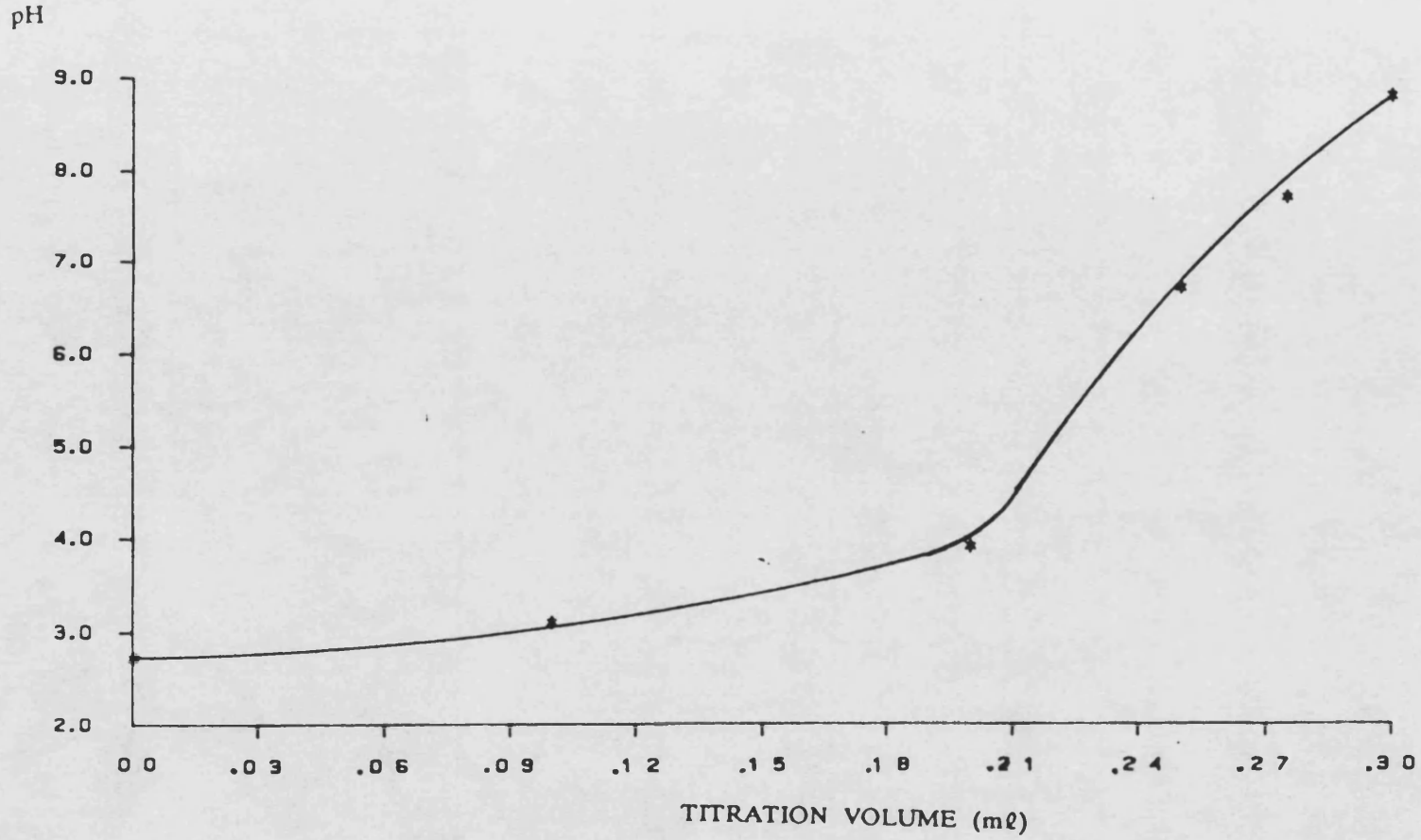
VOL (μl HCl)	pH
0	10.7
50	10.6
100	10.5
200	9.5
225	9.0
250	7.4
260	7.0
270	5.6



Titration curve of epoxide activated cellulose membranes

Titration of iminodiacetic acid activated membranes using 0.1M NaOH.

VOL (μl NaOH)	pH
0	2.7
100	3.1
200	3.9
250	6.7
275	7.7
300	8.8



Titration curve of iminodiacetic acid activated cellulose membranes

NOMENCLATURE

CHAPTER II

AHA - 6-aminohexanoic acid
p-ABA - para-aminobenzamidine
CDI - carbonyldiimidazole
CNBr - cyanogen bromide
EDC - (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)
HCl - hydrochloric acid
IDA - iminodiacetic acid
M - molar
MES - methane-ethane sulphonic acid
mMol - millimoles
γMol - micromoles
NaOH - sodium hydroxide

CHAPTER III

b - concentration of vacant binding sites on adsorbent. (mol/cm^3)
c - concentration of adsorbate in solution.(mol/cm^3)
c_o - initial concentration of adsorbate in solution.(mol/cm^3)
E - molecule of enzyme
EL - molecule of enzyme–ligand complex
k_1 - forward rate constant.(M^{-1}/sec)
k_2 - backward rate constant.(sec^{-1})
K_d - dissociation constant (mol/cm^3)
K_{de} - effective dissociation constant.(mol/cm^3)
L - molecule of ligand
P - proportion of total adsorbate that is bound to adsorbent. (%)
q - amount of adsorbate bound to adsorbent/ unit volume of adsorbent.(mol/cm^3)
q_m - maximum capacity of adsorbent for adsorbate/ unit volume of adsorbent.(mol/cm^3)
tfin- time for the end of the adsorption analysis. (secs)
V - volume of adsorbate.(cm^3)
v - volume of adsorbent.(cm^3)

CHAPTER IV

AIDS - Aquired Immune Deficiency Syndrome.
EDTA - ethylenediaminetetraacetic acid
HSA - human serum albumin
IU - international units
K - kilo
mA - milliamp
min - minute
mg - milligramme
TEMEDA- N,N,N N - tetramethylethylenediamine
Tris - Trizma^R

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